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13. ABSTRACT *(Maximum 200 Words)*

Purpose We have identified and characterized the expression of caveolin-1 (cav-1) a gene related to prostate cancer metastasis and hormone independence. We proposed to elucidate the molecular pathway of castration-induced regression and the mechanism of caveolin-1 mediated hormone resistance. We attempted to develop novel therapeutic approaches for hormone refractory prostate cancer based on this new information.

Scope These studies were designed to confirm the role of caveolin-1 in androgen independent prostate cancer and identify novel therapeutic approaches to androgen insensitive prostate cancer.

Major Findings Expression of caveolin-1 under hormonal regulation. Overexpression of caveolin-1 confers androgen insensitivity and increased cell viability. Suppression of caveolin-1 can restore androgen sensitivity and decrease cell survival/clonogenicity. Caveolin-1 is secreted by mouse and human metastatic prostate cancer cells and may have paracrine and/or autocrine anti-apoptotic activities. Further studies indicate that antibody specific for cav-1 can be used in an orthotopic mouse model for prostate cancer to suppress tumor growth and metastatic spread.

Significance The inhibition of prostate cancer with systemic antibody therapy alone or possibly with other modalities such as chemotherapy may ultimately be of great importance in successfully treating metastatic prostate cancer.

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INTRODUCTION

The natural history of prostate cancer involves progression to metastasis and the eventual development of hormone refractory disease. In normal prostate, removal of androgenic hormones results in regression of the gland involving apoptosis of more than 60% of the luminal epithelial cells. Although often initially sensitive to removal of androgens, prostate cancer cells eventually lose this response and continue to grow and spread in the absence of androgenic steroids. Multiple genetic activities are involved in androgen ablation-induced prostate regression, yet very little is known regarding the rate limiting steps in the molecular cascade that leads to regression or the molecular basis of hormone resistance in prostate cancer. We previously developed a mouse model to identify metastasis-related genes in prostate cancer. This model includes a series of clonal cell lines derived from prostate cancer metastases that developed *in vivo* using the mouse prostate reconstitution model system. One of the gene products we found to be associated with metastasis in this model as well as in human prostate cancer is caveolin. The production of stably selected clones with antisense caveolin-1 resulted in a significant reduction in metastatic activities relative to vector-control clones and parent cell lines. Surprisingly, we discovered that tumors produced by the antisense caveolin-1 clones significantly regressed in response to surgical castration *in vivo*. Eleven days following androgen ablation, tumors derived from three independent antisense clones regressed by approximately 30% relative to the wet weights produced in either vector-control clones or parental clones which did not respond to castration therapy under the same conditions. The antisense caveolin-1 tumors that responded to castration therapy also demonstrated significantly increased levels of apoptosis relative to either vector-control clones or parental cell lines. Therefore, our data indicated that reduction of caveolin-1 levels not only suppressed metastatic activity but also restored androgen sensitivity. We are of the opinion that these novel results establish a new paradigm for understanding androgen refractory disease and open the door for new innovations in prostate cancer therapy. We proposed to further elucidate the molecular pathway of castration-induced regression and the mechanism of caveolin-1 mediated hormone resistance. We attempted to develop novel therapeutic approaches for hormone refractory prostate cancer based on this new information.

BODY

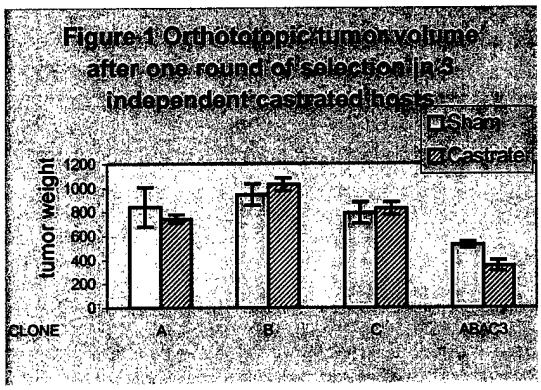
We will provide a detailed account of the research accomplishments for each of the items in the approved statement of work.

STATEMENT OF WORK

(1) To correlate and determine the reversibility of caveolin expression with castration-induced regression in mouse prostate cancer cell lines (months 1–8).

- To complete a selection experiment in which ABAC3 is subjected to a series of growth periods in castrate male animals *in vivo* and analyze AR expression, caveolin expression, and androgen sensitivity following the final round of selection (months 1–8). [24 mice for selection, 128 for evaluation]

We injected the antisense caveolin cell line ABAC3 into three separate mice that had been castrated seven days before. Each of these was then allowed to grow for 14 days as an orthotopic tumor at which time it was removed from the animal weighed and portions collected for immunohistochemistry and portions placed in tissue culture. After a brief period of in vitro growth the cells were collected and a single cell suspension reinjected for orthotopic tumor growth in either castrate or intact animals. A portion of the cells was also lysed and Western blotting performed to evaluate caveolin and AR protein expression levels. The tumors were allowed to grow for 14 days and the entire procedure repeated. Thus cells were grown for three cycles in castrated animals and the tumors evaluated at each cycle for androgen sensitivity and AR and caveolin expression. The results of tumor growth at the first round are presented in Figure 1. The antisense caveolin clone ABAC3 quickly reverted to androgen insensitivity in the three experimental groups (see Figure 1). All of the clones also reacquired expression of caveolin (Figure 2). The expression of AR was also regained.



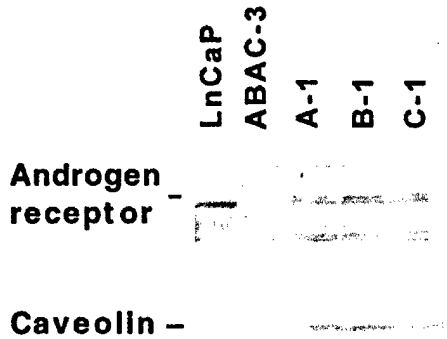


Figure 2. Western blot of cells grown from three independent injections (A-C) for one round of ABAC3 orthotopic tumors grown in castrated animals. Equivalent amounts of protein Western blotted and reacted with antibody against androgen receptor (top) or caveolin-1 (bottom). Control LnCaP and ABAC3 cell extracts showed minimal caveolin protein but the ABAC3 cells re-acquired expression of caveolin-1 after passage in the castrated mice. Expression of androgen receptor was also increased by growth in castrated animals.

- To supertransfect antisense caveolin clones (ABAC3, ABAC5, and BACS4) with sense-caveolin Babepuromycin vector and determine androgen sensitivity of selected stable clones *in vivo* using established hormone manipulation conditions (months 1–8). [288 mice]

We established that the antisense clone ABAC3 rapidly reverted to androgen insensitivity and gained the re-expression of high levels of caveolin after a short time *in vivo*. This result essentially answered the question raised in this specific set of experiments in a highly stringent *in vivo* system. This system is highly preferred to the super transfection approach which can result in considerable clonal selection that can obfuscate interpretation of the results.

- To complete immunostaining of all tissues and quantitate results as well as establish cell lines for future studies (months 1–8).

We have completed the staining and are continuing with the quantitation. Representative immunohistochemical results are presented below (Figure 3).

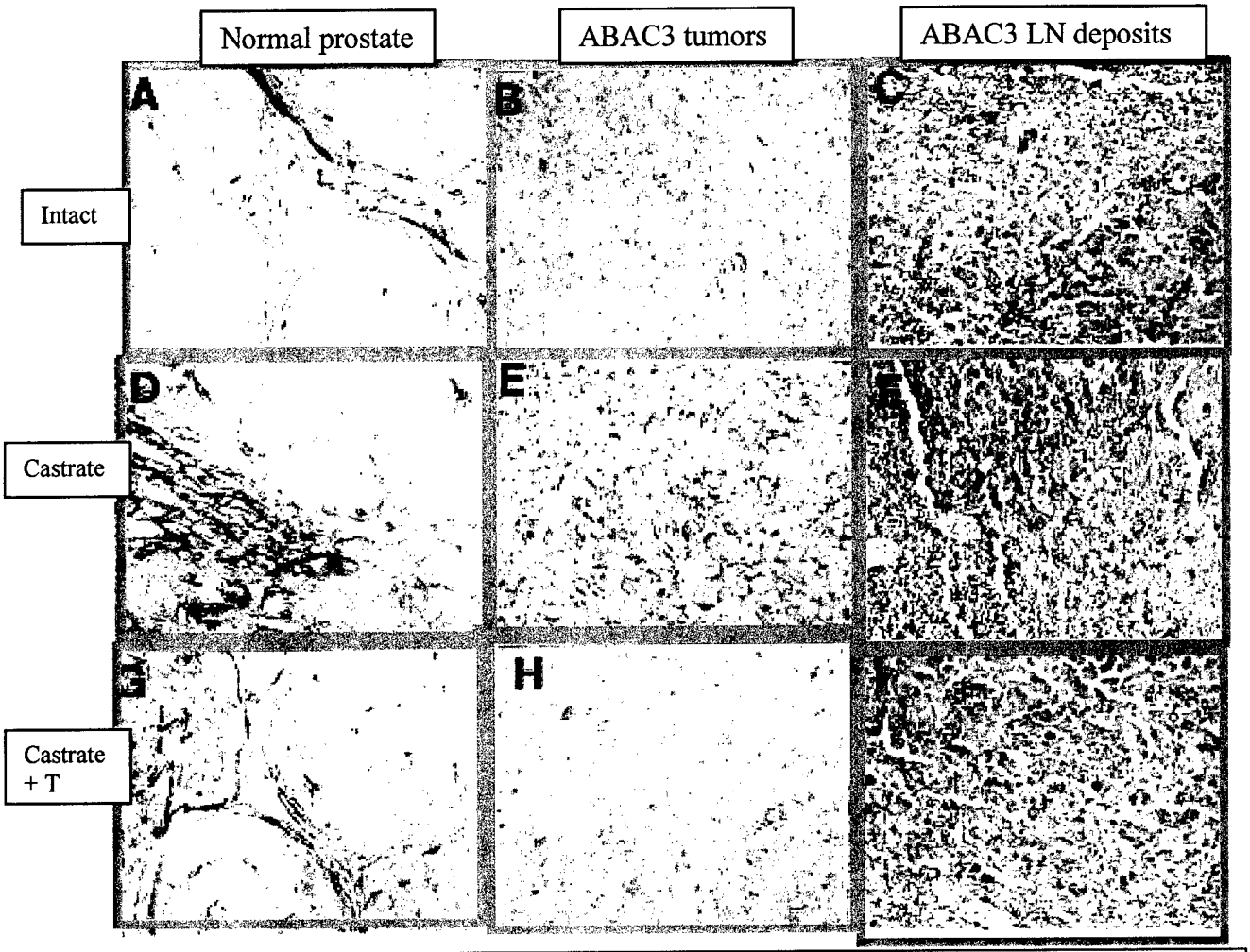


Figure 3. Immunohistochemical detection of caveolin-1 in tissue from normal intact mice (top row) castrated mice (middle row) or castrated mice that also received an implant of silastic tubing with testosterone (bottom row). The tissues stained were from normal prostate (panels A, D and G), the ABAC3 orthotopic tumors (panels B, E and H) and lymph node metastases from the ABAC3 tumors (panels C, F and I). Caveolin-1 in normal prostate tissue is localized in the stroma. . Note the increased staining of caveolin-1 in the castrated as well as testosterone supplemented ABAC3 tumors and lymph node metastases.

(2) To establish the kinetics of castration-induced regression in regard to bioactivity and gene expression (months 3–16).

- To determine the mitotic index of all independent animals by counting mitotic figures under a high-power microscope (months 1–4).

We have previously reported (annual report, 1999) the level of mitosis as determined by counting mitotic figures in orthotopic tumors. The results indicated the lack of a profound effect of castration upon the mitotic index irrespective of caveolin-1 status.

- To determine the apoptotic index using the TUNEL methodology by staining representative sections of all tissues described in Figure 1 and counting apoptotic bodies using high-power microscopy (months 3–16).

We have previously reported (annual report, 1999) the level of apoptosis as determined by staining sections with TUNEL and counting apoptotic figures in orthotopic tumors. The antisense cell line with the lowest level of caveolin-1, ABAC3, had the most pronounced increase in apoptosis in castrated animals. Increased apoptosis was also observed in ABAC5, the other antisense caveolin-1 cell line derived from 148-1 LMD. The other antisense cell line, BACS4, derived from 151-2 LMC, also displayed increased apoptosis in castrated hosts. This provides a correlation for the reduction in tumor volume in castrated animals with levels of caveolin-1.

- To isolate RNA and perform Northern blotting analysis and perform *in situ* hybridization for a panel of selected genes known to be involved in castration-induced regression response (months 6–16).

We have screened a commercial microarray (Clontech) with mRNA from ABAC-3 and LNCaP cells for changes in gene expression that occurred as a result of testosterone stimulation *in vitro*. This is a preferred method for the questions we are addressing in the proposed work. Commercial microarrays enable us to initially screen many more genes than we otherwise would have using Northern blotting as a first step. Once candidate genes were identified we then proceeded with Northern blotting to confirm the results. Thusfar we have confirmed that cytokeratin-8 and –18 mRNAs are induced by testosterone (data not shown). We are considering future studies that may elucidate their potential role as survival factors and their possible regulation by caveolin-1 in prostate cancer.

(3) To determine the role of NOS and nitric oxide production in castration-induced regression response (months 6–24).

- To stain representative tissues for three NOS forms, including iNOS (months 6–24).

Although this work is in progress we have not yet completed the analysis of the tissues from the castration-induced regression. Additional tissue sections have been processed for staining and will be stained with a commercial iNOS antibody which we have previously shown to be useful for analysis of tumor specimens (Nasu *et al.*, 1999). These studies will be completed at a later date.

- To determine nitric oxide production in frozen tissues and/or frozen tissue sections using the arginine to citrulline conversion technique (months 6–16).

We determined total NOS activity in tumor tissues collected at the time of harvesting (d14) and have detected increased activity in tumors from castrated mice relative to sham operated mice and presented the results in the previous annual report (1999). There was not a difference that related to caveolin levels, as the control cell line ABH14, which did not regress, appeared to have a greater increase in NO production than the antisense caveolin clone ABAC3, which did regress. This may be a reflection of the generation of NO by host cells which have infiltrated the tumor rather than from the tumor per sé. We therefore feel that immunolocalization will provide a more definitive answer to this aim.

- To determine the sensitivity of castration-induced regression mechanism to FK506 *in vivo* using established methodologies (months 6–12). [192 mice]

We have not have yet had the opportunity to pursue these experiments. Before proceeding with these *in vivo* studies we felt it would be more prudent to further analyze gene expression patterns in the presence or absence of testosterone *in vitro* to better evaluate the potential for this drug treatment to impact on specific gene activities and castration-induced regression. Since the *in vitro* studies were completed just prior to expiration of the grant we will pursue these studies in the future independently of DOD funding.

(4) To identify gene activities involved in castration-induced regression using DD-PCR (months 6–30).

- To isolate RNase from tissues previously analyzed as well as control tissues, including all lobes of normal prostate under the specific hormone conditions used previously *in vivo* (months 6–30).

We collected tissues at the time of necropsy and tumors into portions that were either processed for immunohistochemical studies or immediately frozen in liquid nitrogen and stored at –80 for subsequent extraction of RNA and/or DNA. We have purified RNA from selected tumors by extraction with UltraSpec. The purified RNA has been used for subsequent studies described below.

- To perform DD-PCR using a panel of random primers (10-mers) on mRNA and identify fragments that are increased or decreased in expression (months 8–30).

We have used a series of 10-mer oligonucleotides to perform RT-PCR in the presence of ^{33}P d-ATP and separated the reaction products on polyacrylamide gels then visualized the bands by autoradiography. An example of such a gel with just one primer is shown below.

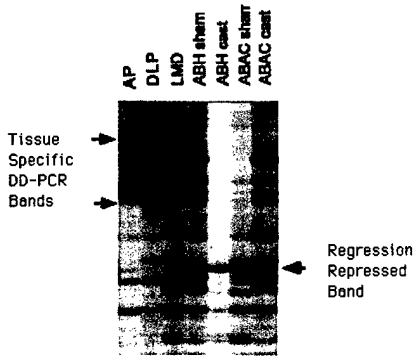


Figure4. DD-PCR analysis with mRNA isolated from orthotopic tumor tissue in sham or castrated mice. Control mRNA from normal 129 mouse anterior prostate (AP) or dorsal lateral prostate (DLP) indicates the ability to detect tissue specific gene expression. The presence of a band which is repressed in the regressing ABAC3 tumor is seen when comparing antisense caveolin clone ABAC3 in the castrated host with the sham host. This same band was present in both sham and castrated mice with the control clone ABH11 or parental LMD tumors.

- To isolate and clone fragments and test clone fragments using a simplified Northern blotting analysis that includes a single sham and castrate vector control clone and antisense caveolin clone (months 9–30).

We have developed *in vitro* systems for hormone manipulation of ABAC-3 cells (as well as LNCaP cells) in parallel with the basic *in vivo* castration model. Our plan is to compare the two systems using both commercial microarray screens and DD-PCR. To this end we have isolated and cloned numerous DD-PCR fragments from the *in vitro* system, screened several of them by Northern blotting and proceeded with DNA sequencing. We have demonstrated that cytokeratin –8 and –18 are upregulated by testosterone and further shown that caveolin-1 may induce these genes. We have also developed a novel approach to screening DD-PCR fragments using a microarray strategy. DD-PCR fragments are eluted from the original gel and re-amplified these are then gel purified and precise amounts blotted onto nitrocellulose membrane using a commercial slot blotting apparatus. These blots can then be screened with mRNA that has been reverse transcribed to cDNA with radioactive label incorporated.

- To test extensively the cloned fragment by extended Northern blotting, including all eight cell lines as well as four control tissues under all hormonal conditions (months 10–30).

As described above we have tested some cloned fragments isolated from the +/- testosterone *in vitro* model for regulation by testosterone. Indeed we have confirmed that cytokeratins-8 and –18 are upregulated by testosterone at the mRNA level. We therefore consider these intermediate filaments may function as "survival factors" or as components of a survival pathway in prostate cancer cells.

- To test selected sequences for cell-specific expression *in vivo* using *in situ* hybridization.

The expression of cytokeratin-8 and –18 has been well characterized in a variety of tissue epithelia. In the prostate these cytokeratins are specific markers for luminal epithelium (the likely precursor of most malignancies). We have proceeded to screen mouse and human primary versus metastatic lesions with specific commercially available antibodies and found both cytokeratin 8 but not 18 is more highly expressed in a majority of human specimens from metastatic deposits versus primary tumors.

- To clone the full-length cDNA for genes found to be involved in castration-induced response using cDNA libraries that have been previously prepared for 148-1LMD and 148-1PA cell lines (months 8–30).

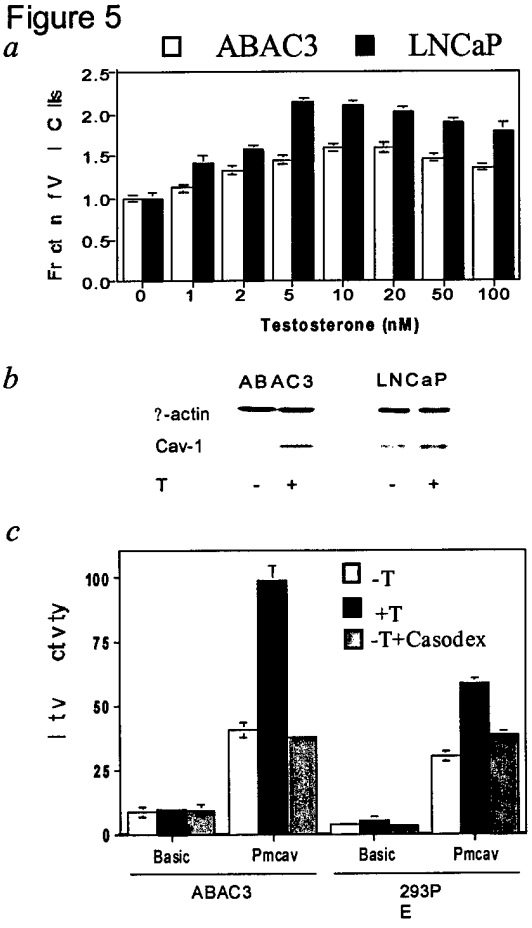
We have not yet obtained the full-length cDNAs for cytokeratins-8 or –18 in expression vectors or retrovirus. We will consider pursuing the overexpression of these "survival factors" in the *in vitro* models described below.

(5) To establish in vitro models for androgen sensitivity using selected high and low caveolin producing mouse and human prostate cancer cell lines.

- To determine the response to testosterone in regard to cell growth *in vitro* using all eight mouse prostate cancer cell lines, including parental cells, vector control clones, and antisense caveolin clones and selected human prostate cancer cell lines (ND-1, PC-3 and LNCaP) *in vitro* (months 15–30).

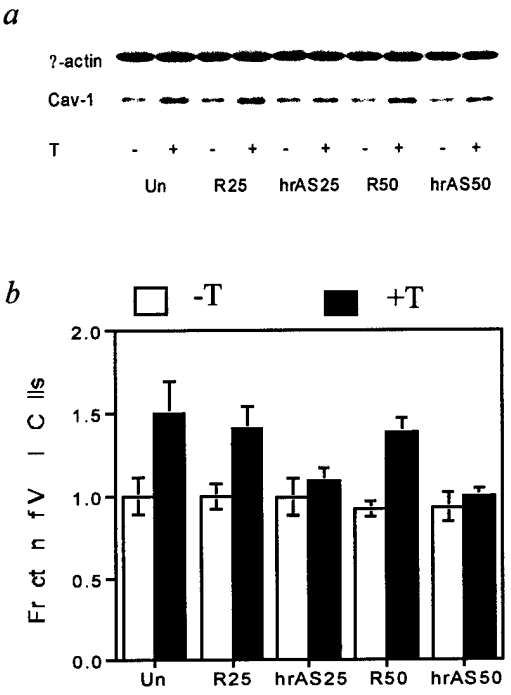
We have completed an intensive evaluation of the androgen responsiveness of mouse and human cell lines with an emphasis on caveolin-1 expression and recently had this manuscript accepted for publication in Cancer Research (see Appendix for attached manuscript for complete results and details). In brief, we concentrated on ABAC3 and LNCaP cell lines because they have low to undetectable levels of caveolin-1. In Figure 5a we demonstrate the testosterone dose range for enhanced viability in an *in vitro* assay in which 100-200 cells are seeded in a well of a 96 well plate in serum free medium with or without testosterone. After three days MTT is added to the well and after short term incubation live and dead cells are counted to determine the viable fraction. To provide two independent measures of viability we have measured the level of ATP in cells treated as above using a commercial assay (ATPlite, Packard Instruments) and reseeded cells from the serum free conditions of the 96 well plate to 10 cm dishes with complete growth media to determine the number of cells capable of forming colonies after the three day testosterone deprivation (see figure 1b and c in attached manuscript in appendix). Both of these assays confirmed the validity of the MTT assay and the observation that testosterone upregulation of caveolin-1 promoted cell survival. We also evaluated the effects of testosterone on expression of caveolin-1 protein by western blotting (Fig 5b) and found that testosterone promoted increased

Figure 5



cell survival in parallel with an increase in the level of caveolin-1 protein (a more extensive evaluation confirmed the dose dependency of expression in ABAC3 and LNCaP as well as in a androgen responsive mouse primary tumor cell line 148-1PA (see appendix Li *et al.,* 2001 figure 2). To determine whether upregulation of caveolin-1 by testosterone occurs at the transcriptional level, a luciferase reporter vector under the transcriptional control of the mouse caveolin-1 promoter, pGL3-Pmcav-1-luc, (Timme et al, 2000) was used. Forty-eight hours after transfection, ABAC3 or 293PE cells were split, re-seeded at low density ($1x10^5$ cells per 15 cm plate) in SFM or SFMT, and grown at 37°C with 5% CO_2 for 24 hours prior to the luciferase assay. The relative activity of luciferase in ABAC3 cells was increased more than 2-fold by testosterone (filled bars, Fig. 5*c*). Similarly, the relative activity of luciferase in 293PE cells (androgen receptor (AR) positive) was also increased approximately 2-fold control levels by testosterone (Fig. 5*c*). This enhanced activity could be blocked by the addition of 1μM of Casodex, a direct antagonist of the AR (shaded bars, Fig. 8*c*) indicating that the upregulation of caveolin-1 by testosterone is mediated by AR. We have also determined a two fold response of the caveolin-1 promoter in LNCaP cells with this reporter construct (Fig. 3 in Li *et al.,* 2001 in appendix).

Figure 6 Antisense caveolin adenovirus infected ABAC3



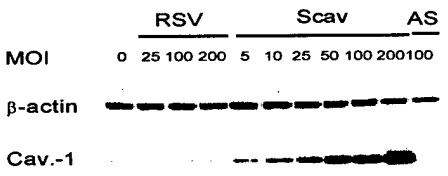
To further correlate caveolin-1 expression with androgen responsiveness we infected ABAC3 cells which express low levels of caveolin-1 with an adenovirus expressing the antisense orientation of the human caveolin-1 cDNA at an MOI of either 25 or 50 (AS25 and AS50) compared to control virus without the antisense caveolin-1 construct (R25 and R50), followed by testosterone stimulation. The results in figure 6*a* show that antisense human caveolin-1 abrogated the up-regulation of caveolin-1 by testosterone. As a consequence of reduced caveolin-1 expression, the survival effects of testosterone were significantly reduced when evaluated by the MTT assay (Fig. 6*b*). We have also used the clonogenic assay to further evaluate the reduced survival associated with caveolin-1 downregulation with the AS virus treatment (Fig. 4 in Li *et al.,* 2001 in appendix). In the clonogenic assay the R25 and R50 infected cells had a 2-3 fold increase in colony number when treated for three days with testosterone compared to without testosterone or when compared with the AS virus with or without testosterone.

To determine whether caveolin-1 could promote survival in human prostate cancer cells in the absence of testosterone, we infected LNCaP cells with an adenoviral vector expressing human sense caveolin-1. Dose-dependent expression of human caveolin-1 was induced by increasing

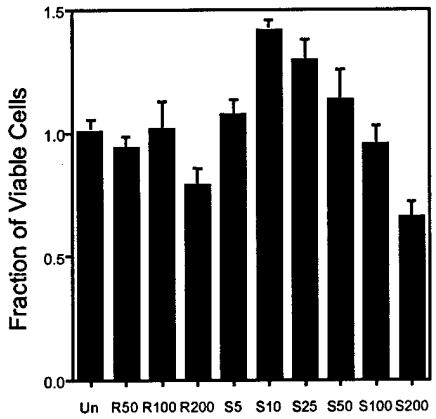
multiplicities of infection (MOI) of the recombinant adenoviral vector. The results demonstrated that the induction of caveolin-1 was detectable at MOI = 5, and gradually increased with increasing MOI (Fig. 7*a*). As expected, caveolin-1 provided significant survival protection to LNCaP cells from growth factor/survival factor depletion-induced conditions. Surprisingly, the maximum protection of caveolin-1 was observed at MOI= 10 (S10, Fig. 7*b*), which represents only a modest level of caveolin-1 (Fig. 7*a*). While survival protection ranged from MOI 5-50, the protection eventually diminished at MOI = 100 and at MOI = 200 reduction of viability was observed (Fig. 7*b*). We confirmed these results with an independent measure of viability, the ATPlite assay (Fig. 5C in Li *et al.*, 2001 in appendix). We have also conducted an evaluation of clonogenic survival following infection with the sense caveolin-1 virus or control virus. LNCaP cells were infected and kept in serum free media for three days with or without testosterone. The cells were then transferred to 10 cm plates with complete growth medium (RPMI1640 + 10% FCS). Colonies which formed were enumerated and confirmed that overexpression of caveolin-1 with an adenoviral vector during a three day androgen depletion restored viability when the dose of vector was in a similar range of MOI's as that used for the MTT assay depicted above in figure 7b.

Fig 7 Caveolin-1 adenovirus in LnCaP

a



b



- Depending on the results of the studies outlined above, to repeat experiments involving both kinetics of androgen-induced regression regarding bioactivity in gene expression, the measurement of NO acid activities and NO production, and FK506 sensitivity *in vitro* (months 15–30).

The *in vitro* studies that would inform these experiments have just been completed and acccepted for publication with adequate and meaningful insight into the kinetics of androgen induced regression (Li *et.al.*, 2001) therefore the proposal to measure NOS activities or FK506 sensitivity will be pursued in future studies.

(6) To produce transgenic mice that overexpress caveolin cDNA in prostate and to test these mice for abnormalities and androgen sensitivity (months 1–30).

- To establish 2–3 founder lines of transgenic mice and confirm expression of the caveolin transgene as well as caveolin levels using immunohistochemistry (months 5–12). [36 mice]

In collaboration with Dr. Franco DeMayo we have injected the caveolin transgene into oocytes and implanted these to attempt to establish founder lines expressing the caveolin-1

gene in a tissue specific fashion. We have screened about 50 offspring for the presence of the transgene. The mice which were positive for the transgene by PCR on tail DNA were raised and bred to develop potential founders. Each was then tested for possible transmission of the transgene and tissue specific expression. In the 1999 annual report we presented a figure to demonstrate RT-PCR on tissues from several F1 mice. Unfortunately, mouse 8042, a male with a probsin-caveolin-1 construct did not produce any other offspring. Just recently we identified two more mice which appear to have good prostatic expression (#'s 9087 and 1660). In other F1 offspring the expression appears to more prominent in brain than in mammary gland of females. We will continue to analyze these mice and generate more F1 mice from the founder of 9087 We have however identified two additional founder mice #8489 and #8041. We have successfully bred these and have offspring positive for the transgene (by analysis of tail DNA). There are currently 13 mice that are 12 months old, 16 mice between 6 and 12 months old, and 54 mice less than 6 months old. The offspring are and a mix of male and female mice derived from both founders.

- To determine the developmental abnormalities in close consultation with Dr. Luan Truong in transgenic mice (months 7–12). [24 mice]

We will continue these experiments. Any results that are published based on these results will cite the DOD as a funding source. The animals will be sacrificed and analyzed for any developmental abnormalities based on analysis at gross necropsy and careful evaluation of the hematoxylin and eosin stained sections of various tissues collected at necropsy.

- To determine pathological correlates of abnormalities in association with Dr. Luan Truong (months 7–30).

We will continue these experiments. Any results that are published based on these results will cite the DOD as a funding source. The animals will be sacrificed and analyzed for any detectable pathological abnormalities by careful visual observation and collection of tissues for paraffin embedding and sectioning followed by hematoxylin and eosin staining. Dr. Truong will assist in the pathological analyses.

- To determine the androgen sensitivity *in vivo* of transgenic mouse prostate and genitourinary tissues (months 15–30). [96 mice]

Since we now have two founder mice we will continue breeding these, as stated above any published reports will acknowledge the DOD as a funding source for these animals.

(7) To test therapeutic strategies for antisense caveolin *in vivo* and *in vitro* using a series of adenoviral vector systems as well as other therapeutic tools (months 15–30).

- To use established adenoviral vector systems, including PCA3 and PAD-12 shuttle vector systems to produce sense and antisense caveolin recombinant adenoviral vectors and test these vectors *in vivo* for potentiation of androgen sensitivity (months 15–30). [192 mice]

We have generated and purified high-titer adenoviral vectors that encode either the human or the mouse caveolin-1 cDNA in both sense and antisense orientation. The adenovirus have either the CMV or RSV promoter driving transcription. In addition we have purified vectors without the caveolin-1 inserts to use as control. We have made extensive use of the RSV driven promoter constructs in both sense and anti-sense orientation along with the parental RSV control adenoviral vector for the studies to be published in Cancer Research (Li et al., 2001) [manuscript attached in appendix].

- If available from Statement of Work (Item 5) to use, to use both mouse and human prostate cancer cell line to test adenoviral vector systems for the capacity to potentiate androgen sensitivity (months 20–30).

We have completed an intensive evaluation of the ability to potentiate androgen responsiveness of mouse and human cell lines with adenovirus and had this manuscript accepted for publication in Cancer Research (see attached manuscript for results and details). These studies are also elaborated upon above in section (5).

- To establish additional therapeutic objectives, including antisense oligonucleotides as well as retroviral vector systems, as therapeutic tools (months 15–30).

We have attempted to use antisense S-oligonucleotides *in vitro* to decrease the expression of caveolin-1 in selected cell lines. We have used several concentrations of oligonucleotide and several different methods to deliver the oligonucleotide into the cells, e.g. various transfection reagents and conditions. Although we have seen transient reductions in caveolin-1 protein by Western blotting we were unable to establish conditions to reproducibly decrease caveolin-1 levels to the extent obtained in the antisense clones such as ABAC3.

- To explore the possibility of using small molecules and antibodies as therapeutic tools, depending on the results of mechanistic studies (months 20–30).

Considerable effort has gone into identifying the role of caveolin-1 in survival during castration-induced regression and we have been gaining some insight into the genes that are active in response to this pathway and in response to testosterone. Our goals we perhaps overly ambitious to a candidate genes/pathways where commercially available small molecules or antibodies have been developed to block at discrete specific steps in these pathways. We also pursued studies not proposed in the original application that addressed this issue and will present the results of these studies below:

Observations by us independent of this grant and by Richard Anderson at UT Southwestern (Liu *et al*, 1999) suggested that cav-1 could be secreted. We analyzed cav-1 immunohistochemically in a series of Stage D prostate cancer primary tumors and their associated metastases from hormonal treated men and compared to non hormonally treated men. The results are presented in the following table.

TABLE1. CAVEOLIN-1 EXPRESSION IN STAGE D PROSTATE CANCERS IN RESPONSE TO HORMONAL TREATMENT.

Hormone Treatment	Frequency of caveolin-1 positive cancers % (*n*)		Percentage of caveolin-1 positive cancer cells Mean (± s.e.)	
	Primary	Metastases	Primary	Metastases
No	38 (19/50)	62 (34/55)	18.6 (2.4)	35.5 (6.0)
Yes	73 (8/11)*	82 (27/33)*	29.9 (5.9)**	38.1 (4.8)

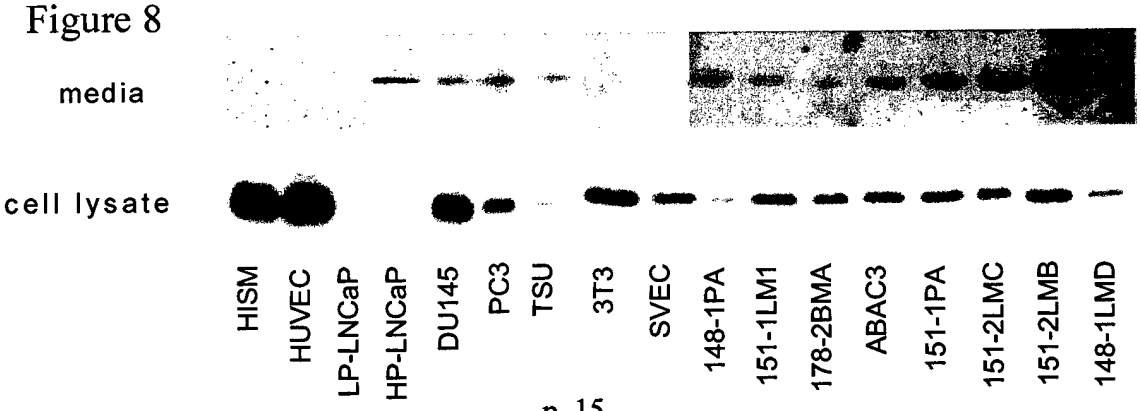
* $P<0.05$, (χ^2 test)** $P<0.05$, (Mann-Whitney test). n = number of patients.

Our previous studies using a distinct set of tissue specimens demonstrated that the frequency of cav-1 positivity was 8% in normal glandular epithelia; 29% in primary cancers with nodal metastases; and 56% in the nodal metastases per sé (Yang et al., 1998). In this study, the frequency of cav-1 positive primary prostate cancers was increased from 38% in the hormonally naive patient group to 73% in the hormone refractory patient group ($p<0.05$, χ^2 test). Cav-1 positivity was demonstrated in 62% of the metastatic specimens from patients who had not been treated with hormone therapy and this frequency was also significantly increased to 82% of metastases from patients treated with hormones ($P<0.05$, Mann-Whitney test).

Further analysis demonstrated that the percentage of cav-1 positive cells was significantly increased from 18.6% in primary tumors to 29.9% in hormone treated primary tumors ($P<0.05$, Mann-Whitney test). The percentage of cav-1 positive cells in metastatic specimens of treated patients was higher (38 %) than specimens from untreated patients (35.5%) but this increase was not significant ($P>0.05$, Mann-Whitney test. It was of interest that the highest percentage of cav-1 positive cells documented (hormone refractory metastases) approached but did not exceed 40%. These results prompted us to investigate the possibility that cav-1 is secreted by prostate cancer cells and functions as a paracrine/autocrine factor.

Cav-1 was detected in conditioned media from androgen-insensitive mouse (151-1LM1, 178-2BMA, 151-2LMC, 151-2LMB, 148-1LMD), and human (DU145, PC3 and TSU-Pr1) prostate cancer cells in variable amounts. In androgen-sensitive, low passage LNCaP cells (LP-LNCaP) cav-1 was not expressed, yet in high passage LNCaP cells (HP-LNCaP) that had reduced androgen-sensitivity cav-1 was expressed and secreted into the medium. In contrast, non-prostatic cells such as endothelial, fibroblast, and smooth muscle had a substantial

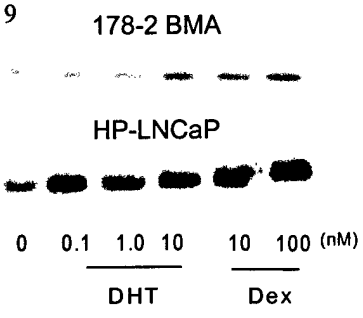
Figure 8



amount of intracellular cav-1 yet minimal or nondetectable levels of cav-1 in their conditioned media (Fig. 8).

We used the mouse prostate cancer cell line, 178-2BMA, derived from a bone metastasis generated from the metastatic mouse prostate reconstitution model and HP-LNCaP to test the possible regulation of cav-1 secretion by dihydrotestesterone (DHT) and Dex *in vitro*. Both cell lines were shown to be insensitive to androgen *in vitro*, i.e., no significant changes in cell number or viability were detected under serum free conditions in the presence and absence of 10 nM testosterone (data not shown). The results showed that cav-1 (21 kDa) was secreted by 178-2BMA cells in response to these steroid hormones, reaching the highest levels at 10 nM DHT, and 100 nM Dex (Fig. 9). The increase in secreted cav-1 in response to these secretagogues was paralleled by a decrease in intracellular cav-1 (not shown). A similar pattern for cav-1 secretion was observed in HP-LNCaP cells (Fig. 9).

Figure 9



We investigated the secretory route for cav-1 by expressing human cav-1 in cav-1 negative LP-LNCaP cells. Following transfection, a substantial amount of ectopically expressed cav-1 was detected in the media compared to that in the cell lysate, and cav-1 secretion was increased in response to DHT. Cav-1 was not detected in the media or cell lysate of the vector control transfected cells, yet all transfected cells excreted prostate specific antigen (PSA) into the media in a DHT regulated fashion (Fig. 10). These results show that cav-1 is secreted by androgen-insensitive mouse and human prostate cancer cells in response to specific steroid hormones. Although, we do not provide evidence for the mechanism by which cav-1 enters the secretory pathway, the results show that ectopically expressed cav-1 is secreted by LNCaP cells and that secreted cav-1 migrates on SDS-PAGE similarly to that derived from endothelial cells and fibroblasts, suggesting that the secreted form is not modified post-transcriptionally. To determine whether cav-1 could also be secreted by human prostate cancer cells *in vivo*, we fractionated human serum and analyzed various fractions for cav-1. Our results revealed that cav-1 is specifically detected in the serum HDL_3 lipoprotein subfraction and that cav-1 levels may be higher in the serum of prostate cancer patients compared to the serum of normal individuals (Fig. 11).

Figure 10

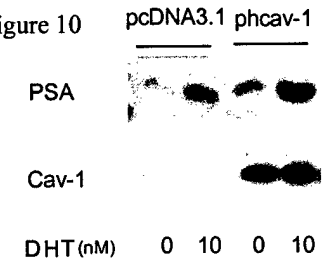
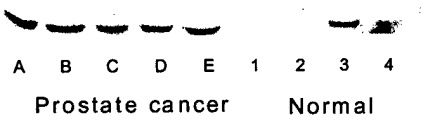
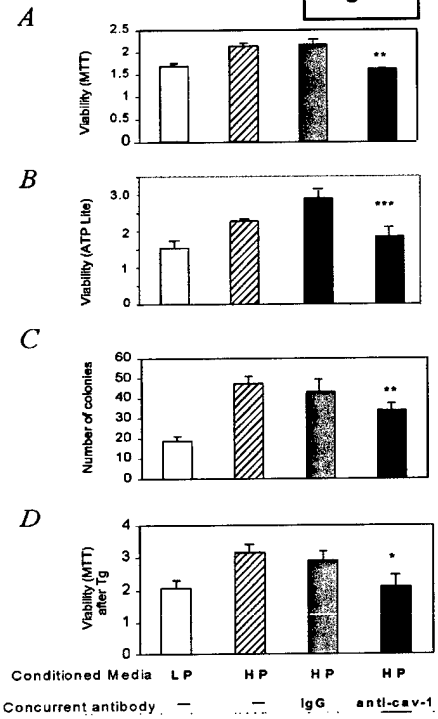


Figure 11



The function of secreted cav-1 was investigated by testing the effects of concentrated conditioned media collected from HP-LNCaP cells on LP-LNCaP cell viability and clonal growth under serum-free conditions. The results indicate that secreted cav-1 was capable of promoting viability, using a standard MTT method (6) (Fig. 12A) or luminescent technique (Packard ATPLite) (Fig. 12B) and of stimulating viability/clonal growth using a clonogenic assay (Fig. 12C). To test whether such activities would be specific for the cav-1 molecule, polyclonal cav-1 antibody was added to conditioned media or rabbit IgG as a control. Treatment of the conditioned media with anti cav-1 antibody reduced the viability significantly ($P<0.001$ for MTT and clonogenic assays and $P<0.0001$ for ATP Lite assay)

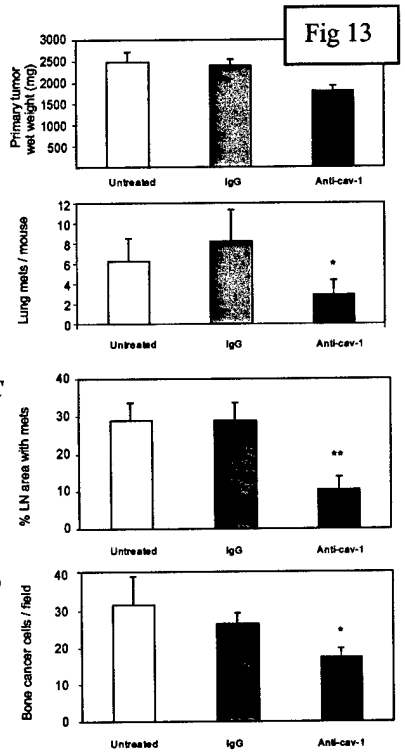
Fig 12



compared to the IgG-treated medium. We further tested the effect of secreted cav-1 on thapsigargin (Tg) induced apoptosis in LP-LNCaP cells. Tg promotes apoptosis characterized by caspase activation and the appearance of apoptotic bodies in these cells (data not shown). The results indicated that secreted cav-1 was able to protect the cells from the apoptotic effects of this drug (Fig. 12D). These studies revealed that media containing secreted cav-1 generates anti-apoptotic activities in prostate cancer cells similar to those elicited following enforced expression of cav-1 within the cell.

We then tested whether blocking secreted cav-1 activity *in vivo* with specific antibodies would result in therapeutic activity potentially through abrogation of the anti-apoptotic effects of secreted cav-1. Androgen-insensitive 178-2BMA cells that spontaneously metastasize with high frequency (nearly 100%) to lung, lymph nodes and bone were grown as orthotopic tumors in adult male mice. After 21 days of treatment with cav-1 antibody or IgG, the animals were sacrificed. The mean tumor wet weight (Fig. 13A) and the mean number of lung metastases (Fig. 13B) of the anti-cav-1 treated group was significantly lower than the IgG-treated group ($P<0.01$ and $P<0.05$, respectively). The cav-1 antibody treated group also had a significantly lower percentage of cancer cell volume in lymph nodes ($P<0.01$) (Fig. 13C). The metastatic cell density in the bone marrow (Fig. 13D) was also reduced significantly ($P<0.05$) in the cav-1 antibody treated mice compared to those of the IgG-treated group. These results show that neutralization of secreted cav-1 *in vivo* by specific antibody suppresses primary prostate tumor growth and spontaneous metastasis to the lung, lymph nodes and bone.

Fig 13



The concept of a secreted autocrine/paracrine factor that directly contributes to androgen resistance in prostate cancer is novel and represents an efficient mechanism for maximizing resistance to various pro-apoptotic stimuli that metastatic cells often encounter during the highly inefficient process of metastasis. Our *in vivo* studies indicating that cav-1 specific antibody delivered intraperitoneally can suppress malignant progression of androgen-insensitive, cav-1 secreting mouse prostate cancer cells are remarkable. These results not only indicate that secreted cav-1 promotes metastasis *in vivo*, but also raise the possibility of using cav-1 as a therapeutic target for androgen-insensitive disease. It is conceivable that when combined with anti-androgen therapy or potentially chemotherapy, cav-1 specific antibody therapy may have greater therapeutic activity. Further studies will be required to address this issue.

KEY RESEARCH ACCOMPLISHMENTS

- Caveolin-1 expression is associated with androgen resistance in prostate cancer
- The establishment of *in vitro* and *in vivo* models for hormone manipulation of prostate cancer cells
- Caveolin-1 is regulated by testosterone at the level of transcription
- Caveolin-1 promotes survival of mouse and human prostate cancer cells *in vitro* models
- The generation of systems and experimental approaches to identify downstream target genes that are affected by caveolin-1 expression and may play a role in cancer cell survival
- A transgenic mouse with prostate specific caveolin-1 expression has been identified.
- Caveolin-1 is associated with hormone refractory prostate cancer.
- Caveolin-1 protein is secreted by prostate cancer cells.
- Secreted caveolin-1 promotes cell survival of caveolin-1 negative prostate cancer cells.
- Antibody to caveolin-1 can block survival promoted by secreted caveolin-1.
- Antibody to caveolin-1 can have anti-tumor and antimetastatic effects in an *in vivo* orthotopic prostate cancer model.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Timothy C. Thompson, Terry L. Timme, Likun Li, Alexei Goltsov and Guang Yang: "CAVEOLIN 1: A COMPLEX AND PROVOCATIVE THERAPEUTIC TARGET IN PROSTATE CANCER AND POTENTIALLY OTHER MALIGNANCIES." *Emerging Therapeutic Targets* **3**(2):337-346, 1999.

Li, L., Wang, J, and Thompson, T.C.: "Dose dependent caveolin-1 protection from thapsigargion-induced apoptosis in prostate cancer cell lines. Abstract #2163 90th Annual meeting American Association for Cancer Research. April 1999.

Likun Li, Guang Yang, Chengzhen Ren, Jianxiang Wang, Michael M Ittmann, Thomas M Wheeler, Terry L Timme, Salahaldin Tahir, Timothy C Thompson. "TESTOSTERONE PROMOTES SURVIVAL OF PROSTATE CANCER CELLS THROUGH UPREGULATION OF CAVEOLIN-1 AND CAVEOLIN-1 IS HIGHLY EXPRESSED IN ANDROGEN RESISTANT HUMAN PROSTATE CANCER." Abstract #2730, 91st Annual meeting American Association for Cancer Research. April 2000.

Likun Li, shin Ebara, Guang Yang, Yasutomo Nasu, Takefumi Satoh, Chengzhen Ren, Jianxiang Wang, Salahaldin Tahir, Terry L. Timme, Alexei Goltsov, and Timothy C. Thompson. Association of PI3-K/Akt pathway and caspase inactivation with caveolin-1 enhanced prostate cancer survival and metastasis. Abstract # 1115, 92th Annual meeting American Association for Cancer Research. March 2001.

Salhaladin A. Tahir, Guang Yang, Shin Ebara, Terry L. Timme, Takefumi Satoh, Likun Li, Alexei Goltsov, Michael Ittman, Joel D. Morrisett, and Timothy C. Thompson. Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. Abstract # 1116, 92th Annual meeting American Association for Cancer Research. March 2001

Salhaladin A. Tahir, Guang Yang, Shin Ebara, Terry L. Timme, Takefumi Satoh, Likun Li, Alexei Goltsov, Michael Ittman, Joel D. Morrisett, and Timothy C. Thompson. "Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer." *Cancer Res.* In press, 2001

Likun Li, Guang Yang, Shin Ebara, Takefumi Satoh, Yasutomo Nasu, Terry L. Timme, Chengzhen Ren, Jianxiang Wang, Salahaldin Tahir, and Timothy C. Thompson: "Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells" accepted for publication Cancer Research 2001.

PERSONNEL FUNDED BY PROJECT

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CONCLUSIONS

We have shown that androgen independent prostate cancer cells with high caveolin-1 can become androgen responsive *in vivo* and *in vitro* by suppression of the expression of caveolin-1. We have shown that caveolin-1 is regulated by testosterone both *in vivo* and *in vitro* in prostate cancer and that caveolin-1 is a principal effector of testosterone-mediated prostate cancer cell survival. The mechanism(s) that underlie caveolin-1 mediated cell survival in prostate cancer are likely complex, but could involve molecular transport and/or signal transduction modulation. Based on our preliminary data they could also involve modulation of the cytoskeleton, specifically alteration in cytokeratin expression and function. We have established novel *in vitro* screening systems as well as *in vivo* and transgenic mouse models for future studies. We were perhaps overly ambitious in our aims as we have not yet completed all proposed studies. Future efforts will be mainly focused on understanding the mechanisms that underlie the capacity of caveolin-1 to sustain survival of prostate cancer cells during metastatic progression and in the absence of testosterone. We have attempted to provide a clearer understanding of the molecular changes associated with caveolin-1 and the androgen resistant prostate cancer phenotype through the identification of genes that lie downstream of caveolin-1. We have shown that caveolin-1 can and serve as a proximal effector of prostate cancer survival. The identification of intermediary molecules in the pathway to hormone resistance may provide a unique target for intervention. We have also developed transgenic mice that overexpress caveolin-1 in the prostate and these mice are going to provide a useful resource to help understand the role of caveolin-1 in prostatic development and prostate cancer as the mice achieve adulthood and can be analyzed or used for future studies. Our *in vivo* studies indicating that cav-1 specific antibody delivered intraperitoneally can suppress malignant progression of androgen-insensitive, cav-1 secreting mouse prostate cancer cells are remarkable. These results not only indicate that secreted cav-1 promotes metastasis *in vivo*, but also raise the possibility of using cav-1 as a therapeutic target for androgen-insensitive disease. It is conceivable that when combined with anti-androgen therapy or potentially chemotherapy, cav-1 specific antibody therapy may have greater therapeutic activity. Further studies will be required to address this issue.

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Nasu Y, *et al.* Adenovirus-mediated interleukin-12 gene therapy for prostate cancer: suppression of orthotopic tumor growth and pre-established lung metastases in an orthotopic model. *Gene Ther* **6**:338-349, 1999.

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Yang, G., *et al.,.* Elevated expression of caveolin is associated with prostate and breast cancer. *Clin. Cancer Res.* **4:**1873-1880, 1998.

APPENDICES

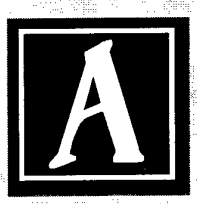
Attached as appendix information are the following publications:

Thompson, TC, Timme, TL, Li, L, Goltsov, A, Yang, G: CAVEOLIN-1: A COMPLEX AND PROVOCATIVE TARGET IN PROSTATE CANCER AND POTENTIALLY OTHER MALIGNANCIES. Emerging Therapeutic Targets **3**:337-346, 1999.

Likun Li, Guang Yang, Shin Ebara, Takefumi Satoh, Yasutomo Nasu, Terry L. Timme, Chengzhen Ren, Jianxiang Wang, Salahaldin Tahir, and Timothy C. Thompson: "Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells" Cancer Research (accepted for publication), 2001.

Salhaladin A. Tahir, Guang Yang, Shin Ebara, Terry L. Timme, Takefumi Satoh, Likun Li, Alexei Goltsov, Michael Ittman, Joel D. Morrisett, and Timothy C. Thompson. Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. *Cancer Res.* (In press), 2001.

Oncologic, Endocrine & Metabolic



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Review

1. Introduction
2. Caveolins and caveolae
3. Consequences of altered caveolin expression in metastatic prostate cancer cells
4. Caveolin/caveolae pathways as therapeutic targets
5. Molecular surrogates of caveolin-1-based therapeutic targets
6. Summary

Acknowledgements

Bibliography

Caveolin-1: a complex and provocative therapeutic target in prostate cancer and potentially other malignancies

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Emerging Therapeutic Targets (1999) 3(2):337-346

1. Introduction

Prostate cancer is the most common cancer diagnosed in adult men in the United States and the second leading cause of cancer deaths [1]. The incidence of prostate cancer increased dramatically during the early 1990s due to the widespread use of prostate specific antigen (PSA) screening, but in recent years has subsequently declined (a likely result of saturation of prostate cancer screening). A slight decrease in prostate cancer mortality has also been observed in recent years with an estimate of 39,200 deaths in 1999 [1]. Curiously, a similar level of decline was also observed for many other malignancies [1]. Current curative therapeutic approaches to prostate cancer, i.e., surgery or radiation, are directed exclusively towards the primary tumour, yet occult metastases are often present at the time of treatment and it is the metastatic spread of the disease to bone and other sites in the body that leads to mortality. The only conventional treatment strategy for metastatic prostate cancer, androgen ablation, is based on experimental observations by Huggins and Hodges over fifty years ago [2]. Although androgen ablation often results in palliative effects, this treatment is not curative. Chemotherapeutic approaches against metastatic cancer have been generally ineffective due to an apparent intrinsic resistance of prostate cancer cells to conventional chemotherapeutic agents [3]. It appears that novel treatment strategies, used alone or as an adjuvant therapy, that are effective against metastatic disease will be necessary to substantially reduce prostate cancer mortality.

1.1 A model system of gene-based therapy for metastatic prostate cancer

We have developed an animal model system for research into the molecular mechanisms of metastasis and development of novel therapeutic approaches for the treatment of prostate cancer metastasis. We refined and extended the *in vivo* metastatic mouse prostate reconstitution (MPR) model system [4] by developing a set of cell lines easily adaptable to molecular methods such as differential display-PCR (DD-PCR) *in vitro* [5,6], but also useful for evaluating metastatic activities *in vivo* [7]. Early passage clonal cell lines were generated from primary and metastatic tumours that developed following initiation of p53 null foetal prostate tissues with the *ras* and *myc*

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oncogenes [8]. Matched pairs of metastasis- or primary tumour-derived cell lines were obtained from cancer tissues in the same animal. These cell lines therefore have an identical genetic background (p53 null foetal prostate tissues initiated with the *ras* and *myc* oncogenes), and in many cases are also clonally related based on Southern blotting evidence that identified unique integration sites of the initiating oncogenic retrovirus (Ziprasmyc 9) [4]. *In vitro* molecular testing of these cell lines has been restricted to early passage cells to avoid the confusing artefacts that could arise from continued passage of cells in tissue culture. For *in vivo* testing of these clonally related cell lines, we adopted an orthotopic model that most accurately reflects their biological activities and metastatic differences [7]. Interestingly, we found that orthotopic tumours produced by metastasis-derived cell lines tended to grow less rapidly but demonstrated greater spontaneous metastatic potential than their matched cell lines derived from the primary tumour [7]. This comparative model system effectively controls for genetic alterations that are unrelated to the metastatic process, and can therefore be used to identify metastasis-related genes for functional analysis. Subsequent evaluation of human prostate cancer tissues has validated the relevance of identified genes to the human disease process.

1.2 Caveolin-1 upregulation in prostate cancer

With the metastatic MPR model system described above we have identified numerous metastasis-related candidate genes. One of the first sequences identified as being upregulated in metastatic mouse prostate cancer using DD-PCR in this system encoded mouse caveolin-1 [5]. Northern blotting confirmed increased caveolin expression in metastasis-derived cell lines relative to primary tumour-derived cell lines. Western blotting with polyclonal and monoclonal caveolin-1 antibodies also confirmed increased caveolin-1 protein in metastasis-derived mouse cell lines and expression in three of four human prostate cancer cell lines. Extensive immunohistochemical analyses of our mouse model for prostate cancer and clinical human prostate specimens obtained at the time of radical prostatectomy revealed minimal caveolin-1 expression in normal prostatic glandular epithelium, with abundant staining in stromal smooth muscle and blood vessel endothelium. Prostate cancer with documented regional spread to lymph nodes had an increased frequency of caveolin-1 positive cells, and lymph node metastatic deposits had markedly increased levels of accumulation of caveolin-1 protein and a granular staining pattern. Additional evaluations of human breast cancer specimens revealed increased caveolin-1 staining in intraductal and infiltrating ductal carcinoma, as well as nodal disease. Caveolin-1 therefore appears to be associated with human prostate cancer progression and is also present in primary and metastatic human breast cancer [5].

2. Caveolins and caveolae

The caveolin-1 gene is a member of a family of three related genes [9]. Caveolins are the major structural proteins of caveolae, specialised plasma membrane invaginations that were first recognised by electron microscopy as non-clathirin coated pits (or 'cave-like' structures) on the cytoplasmic membrane [10]. They have also been characterised as a specific type of membrane microdomain, termed a raft. These are composed of cholesterol and glycosphingolipids, and are involved in organising membrane components for cellular function [11]. Caveolin is a specific protein that is involved in raft domain formation and may function as a scaffolding protein for caveolae [12]; however, caveolin can also be found associated with intracellular membranes such as those of the Golgi complex [10].

2.1 Caveolae structure and cell-specific expression

Caveolae are 50 - 100 nm invaginations of the plasma membrane, usually with a characteristic flask-like appearance in electron micrographs. Caveolae are abundant in endothelial cells, smooth muscle cells and fibroblasts [13]. They are present in most cell types, and are a characteristic feature on the apical surface of many polarised epithelial cells. Because of their unique lipid composition, caveolae are readily isolated as detergent-insoluble glycolipid-enriched domains (DIGs) [14] based on their insolubility in cold Triton X-100. Their high concentration of glycosphingolipids and cholesterol gives DIGs a low density in sucrose gradients and has allowed purification of caveolae-associated proteins. The major protein found in caveolae was termed caveolin, and is a 21 - 22 kDa protein with an intramembrane domain but cytoplasmic amino and carboxy termini [13]. Ectopic expression of the caveolin-1 gene can induce plasma membrane invaginations containing caveolin in cells

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such as lymphocytes, which normally do not have detectable caveolae [15]. This suggests an important role for caveolin-1 in establishing membrane structure and in the formation of caveolae.

The expression of the caveolin gene family has been extensively studied in cell lines [16]. Caveolin-1 and -2 have a widespread expression pattern and are frequently co-localised to the plasma membrane, suggesting the ability to form hetero-oligomeric complexes [16]. All three mammalian caveolin genes contain an invariant domain of eight amino acids in the N-terminal region, and a similar domain is found even in invertebrate caveolin-1 and -2 [17]. Overall, at the amino acid level there is a 58% similarity and 38% identity between caveolin-1 and -2 [9], but specific antibodies are available to distinguish between the two. The genes for caveolin-1 and -2 appear to be localised to the same region of human chromosome 7q31 [18] and the syntenic region of mouse chromosome 6 [19]. Caveolin-3 is a muscle-specific protein, and mutations in the gene have recently been associated with limb-girdle muscular dystrophy [20].

2.2 Regulation of caveolin-1 gene activity in normal cells

In confluent normal human skin fibroblasts, low density lipoprotein (LDL) in plasma was shown to positively regulate the transcription of caveolin-1 mRNA about three-fold within 1.5 h and lead to a similar increase of free cholesterol efflux into the medium. In contrast, oxysterols reduced caveolin-1 mRNA levels and free cholesterol efflux [21]. In a subsequent study, 924 bases of the human caveolin-1 promoter region were sequenced and two functional sterol regulatory elements (SRE) were identified at nucleotide positions 646 and 395 [22]. The site at 395 bp binds SRE binding protein-1 as detected by gel shift analysis. This binding protein is an inhibitor of transcription of the caveolin-1 gene, as opposed to its stimulatory effect on other promoters [22].

2.3 Biological and biochemical activities of caveolin-1

Caveolin-1 was first cloned and characterised because it is a principle substrate for tyrosine phosphorylation by Rous sarcoma virus [23]. There are numerous reports of co-purification or co-localisation of caveolin with proteins that mediate signal transduction, molecular transport and cell-cell communication activities.

2.3.1 Signal transduction activities

Numerous investigators have detected signal transduction-related molecules in association with caveolin *via* co-purification by subcellular fractionation techniques, and visualisation by immunohistochemistry and/or electron microscopy. Shaul and Anderson have recently reviewed these studies [24], and described the four major groups of signalling molecules enriched in caveolae as those involved in G-protein mediated signalling, Ca^{2+}-mediated signalling, tyrosine kinase/mitogen-activated protein kinase (MAPK) signalling pathways, and lipid signalling. Caveolae appear to be a focal point for compartmentalising, organising and modulating signal transduction activities that begin at the cell surface. Given the complexity of each of the different individual signal transduction pathways and the potential for cross-talk and interactions between them, it seems likely that individual cell types will exhibit different responses to stimuli that are potentially mediated by caveolin/caveolae. Cellular context will likely dictate the presence of specific signalling cascades, and caveolins may interact either to stimulate or inhibit signal transduction [9].

2.3.2 Molecular transporter activities

The abundance of caveolae in endothelial cells lead to studies that implicated them in transcytotic pathways for the transport of blood borne macromolecules such as LDL [25]. Folate appears to be taken up by certain cells in a caveolin-mediated process called potocytosis. Receptor-mediated binding of folate is localised in caveolae, which transiently close and release the folate to a cytoplasmic carrier without going through an endocytic pathway [10]. Transport of sterols has also been reported in certain cell types, and caveolin-1 protein has been shown to bind cholesterol [26] and control free cholesterol efflux from cells [21]. These observations may be related to the putative cycle of caveolin movement between the plasma membrane and the *trans*-Golgi network [13].

2.3.3 Cell-cell communication activities

The urokinase-type plasminogen activator receptor (uPAR) is a glycosylphosphatidylinositol (GPI) -linked protein that is an adhesion receptor for vitronectin on the surface of fibroblasts. In migrating cells it is also found at focal adhesions in association with activated integrins. A stable uPAR-integrin-caveolin complex has been detected and may provide a mechanism for localising extracellular activities and generation of an

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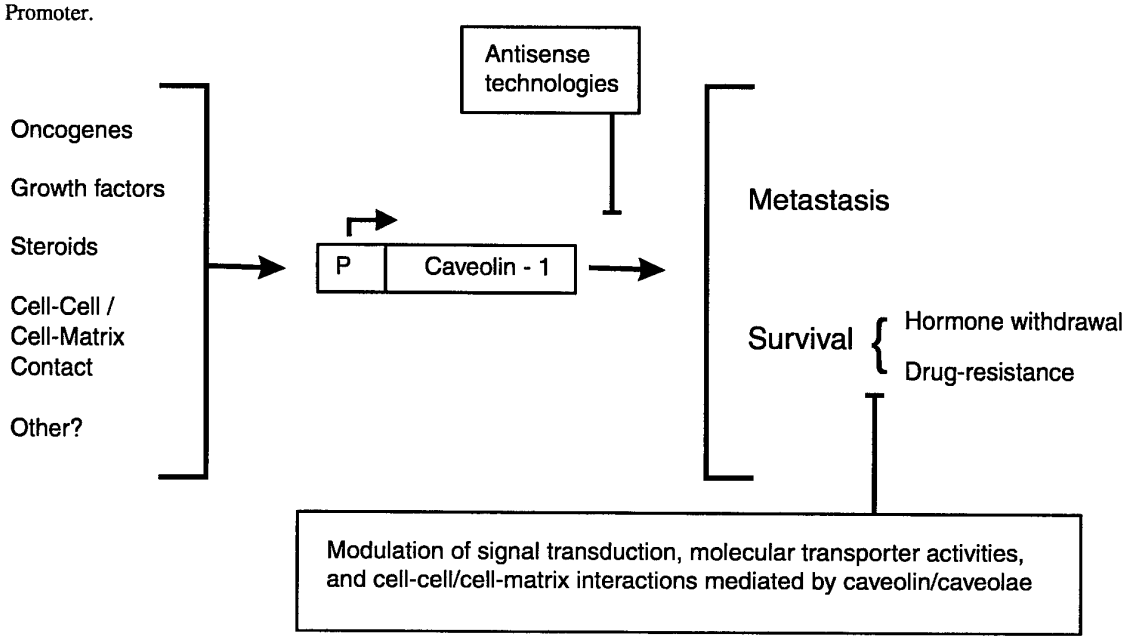
Figure 1: Caveolin-1: a metastasis and hormone/drug-resistance gene.
The central role of the caveolin-1 gene in mediating metastasis and cell survival is depicted. Two possible points of intervention as therapeutic targets are also shown.
P: Promoter.



intracellular signal through integrins [27]. Specific subsets of β 1 integrins have also been implicated in signalling for cell cycle progression and MAPK pathways (linked to the integrins through the adaptor protein Shc and caveolin) [28]. Therefore, the possibility exists for cell-cell and/or cell-matrix initiated, caveolin/caveolae mediated cell signalling that could potentially affect functions related to cell proliferation and cell survival.

3. Consequences of altered caveolin expression in metastatic prostate cancer cells

Through recent studies, a complex picture of caveolin-1 expression and function in malignant cells is emerging. At first consideration, some results appear contradictory. However, a careful analysis of the experimental details of specific studies, including model systems, cell types and physiological context, may resolve apparent inconsistencies. The proposed central role the caveolin-1 gene may play in these processes is discussed below and presented as a schematic summary in **Figure 1**.

3.1 Caveolin expression in transformed cell lines

Caveolin-1 was identified as a major phosphorylation substrate for the v-*src* oncogene [29]. Downregulation of the expression of caveolin-1 has been reported in NIH3T3 cells transformed with activated *abl* or *ras* [30] and *neu* [31] oncogenes. We have also observed decreased caveolin-1 mRNA and protein in *ras*+*myc* transformed NIH3T3 cells [5]. In contrast, the level of caveolin-2 does not appear to be affected by oncogenic transformation [16]. Further observations in transformed NIH3T3 cells indicated that induced expression of caveolin-1 could lead to abrogation of growth in soft agar, suggesting that caveolin may suppress transformation [32]. In addition, it was recently demonstrated that caveolin-1 transfection resulted in growth suppression of selected human breast cancer cell lines *in vitro* [33]. These observations appear to contradict our report that caveolin-1 is overexpressed in metastatic prostate cancer in both mice and humans and in human breast cancer [5]. However, significant differences in gene regulation are likely to exist between *in vitro* transformed fibroblasts and metastatic epithelial-derived tumours *in vivo*. Furthermore, the selection pressure for growth-related properties may be fundamentally different during tumourigenesis *versus* metastasis, and growth suppression functions may accompany other

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over-riding functions related to the survival of metastatic cells *in vivo*. It is clear that considerably more research is needed to understand the growth-related versus metastasis-related activities of caveolin-1, and that these activities must be placed into a relevant cellular context.

3.2 Caveolin-1 overexpression in metastatic mouse prostate cancer cells is associated with androgen resistance

As discussed above, the results of analysis of caveolin-1 expression in both mouse and human tissues, *in vitro* and *in vivo*, indicated that caveolin-1 was upregulated in metastatic prostate cancer [5]. The functional significance of caveolin-1 overexpression in metastatic prostate cancer was explored using metastatic, androgen-insensitive mouse prostate cancer cell lines. We generated a series of stably transfected antisense caveolin-1 clones with reduced levels of caveolin-1 protein relative to vector controls and parental cells. Surprisingly, antisense caveolin-1 tumours generated in an orthotopic model system regressed following castration, whereas vector control and parental cell tumours did not [34]. Furthermore, when a representative antisense caveolin-1 clone was selected for androgen insensitivity by continued growth in a castrated male host, the cells isolated from the resulting tumour were androgen insensitive *in vivo* and demonstrated high caveolin-1 and androgen receptor expression. We also developed an *in vitro* system to further investigate the response of antisense caveolin-1 clones to hormone withdrawal, and observed that antisense caveolin-1 clones underwent significant apoptosis in the absence of testosterone whereas vector control clones and parental cell lines did not. Infection of a representative androgen sensitive cell line with an adenoviral vector to generate relatively high levels of caveolin-1 expression converted the cells to an androgen insensitive phenotype, confirming the association of caveolin-1 protein with androgen insensitivity [34].

3.3 Caveolin-1 overexpression is associated with drug resistance

Two recent papers, published shortly after our identification of caveolin-1 overexpression in prostate cancer [5] and its association with androgen insensitivity [34], provided strong correlative evidence that caveolin-1 is also associated with the drug-resistant phenotype. The multiple drug-resistant human colon carcinoma cell line, HT-29-MDR, and human breast cancer cell line, MCF-7 AdrR, demonstrated significant caveolin upregulation independent of P-glycoprotein expression [35]. *In vitro*-generated taxol- and epithilone B-resistant human ovarian and lung carcinoma cell lines, as well as the vinblastine-resistant SKVLB1 ovarian cancer cell line, also demonstrated significant caveolin-1 upregulation [36].

Caveolin-1 upregulation therefore appears to be associated with multiple drug-resistant phenotypes. In general, these data are congruent with our previous reports of an association of caveolin-1 upregulation with androgen resistance. Indeed, the two sets of data in regard to androgen and drug resistance suggest a possible overlapping common function for caveolin-1 in cell survival/protection.

Although at this point relevant data are limited, certain concepts can be considered regarding caveolin-1 expression in the androgen and drug-resistant phenotypes. Because caveolin-1-positive cells were found at high frequency in metastatic deposits within lymph nodes of human prostate cancer patients that had not been treated with anti-androgens or other drug therapies, it can be inferred that these cells were selected for metastasic potential based on naturally occurring environmental conditions in the metastatic pathway. This consideration, together with recent experimental data that demonstrate a selection advantage of high caveolin-1 cells for resistance to androgen ablation-induced apoptosis [34], leads to an explanation for co-selection for metastasis and androgen resistance based on common features of the metastatic and the castration-induced environment. The levels of testosterone, a steroid growth/differentiation factor, are abruptly reduced upon castration from the extremely high levels that occur within the normal prostate to relatively low levels [37]. This reduction in testosterone can also lead to significant alterations in the expression of growth factors that are under androgenic control [38] (e.g., epidermal growth factor [39]). Interestingly, under conditions of 'normal' prostatic growth factor and androgen levels when potentially metastatic cells are seeded into either the lymphatics or general vasculature, they encounter similar environmental stresses to those that exist within the prostate gland following castration. Because of the presence of various plasma proteins that can bind steroids [37], when potentially metastatic cells enter vascular compartments they likely encounter an abrupt reduction in testosterone, as well as other growth factors. Other selection pressures which the potentially metastatic cells encounter are

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hypoxia and loss of appropriate cell-cell and cell-matrix interactions. These conditions are also similar in many regards to selective pressures encountered in the prostatic environment upon castration [40]. Thus, cancer cells that seed into the systemic circulation during metastatic progression may be selected for survival in a metastatic environment based on properties that overlap with those necessary for survival in a castrated environment.

4. Caveolin/caveolae pathways as therapeutic targets

Thus far, our laboratory has demonstrated that caveolin-1 is associated with both the metastatic phenotype and the androgen-resistant phenotype in prostate cancer. Subsequent reports from other laboratories suggest that caveolin-1 overexpression may lead to drug resistance in various malignant cells. In the previous discussion, we introduced the concept that selection for the metastatic phenotype may involve co-selection for androgen resistance. Interestingly, the potential biological and biochemical activities of caveolin-1 form the basis for rational therapeutic strategies that could impact on both the androgen-resistant and drug-resistant cancer cell.

4.1 Signal transduction targets

A potential mechanistic link exists between the response of prostatic epithelial cells to androgen ablation and specific molecular signal transduction activities associated with caveolin/caveolae. Within one day following androgen ablation, *in vivo* intracellular Ca^{2+} levels rise within prostatic glandular cells and drive intranuclear Ca^{2+}/Mg^{2+}-dependent endonuclease activities contributing to widespread apoptosis [41,42]. Following on from our previous discussion, one might speculate that Ca^{2+} dysregulation in potentially metastatic cells contributes to the selection of the fully metastatic cell that survives or adapts to these pressures. Interestingly, it has been demonstrated in various non-prostatic cells that caveolae are involved in Ca^{2+} transport, storage and Ca^{2+}-mediated signalling [24]. Although the precise levels at which caveolin/caveolae regulate Ca^{2+}-mediated signalling are largely unknown, it is tempting to speculate that this pathway is involved in androgen/drug resistance. Interestingly, first generation modulators of P-glycoprotein include Ca^{2+} channel blockers and calmodulin inhibitors [43,44]. In addition, many drugs (including taxol) can activate a variety of signal transduction pathways that could be subverted or blocked by caveolin/caveolae-mediated cell signalling. Numerous studies have demonstrated that caveolin/caveolae may regulate multiple signal transduction pathways, including those related to nitric oxide synthase, MAPK and lipid signalling molecules [12,24].

4.2 Molecular transporter targets

The reported association of overexpression of caveolin-1 with drug resistance is consistent with a cell protection/survival function for caveolin/caveolae. Interestingly, there are functional similarities between caveolin-1 and the well-established drug resistance gene, P-glycoprotein [45], that are worthy of discussion. P-glycoprotein is a contributing factor to multi-drug resistance in numerous malignancies [46]. It is a plasma membrane ATPase- and energy-dependent drug efflux pump, and these specific properties are responsible to a large extent for drug resistance in cells that overexpress the gene [47]. These activities can be directly compared to the molecular transport properties of caveolin which has been shown to transport intracellular cholesterol to the cell surface [21]. It is conceivable that this functional aspect of caveolin-1 is somehow related to the extensively studied P-glycoprotein molecular transport activities in regard to drug resistance [46]. Modulators of P-glycoprotein have been developed with the intent of enhancing the efficacy of anticancer drugs without significantly altering the pharmacokinetic interactions of the anticancer agent [48]. In some cases it has been shown that these modulators can inhibit drug efflux, thus establishing the importance of this P-glycoprotein-related activity as a target for drug development. By extrapolation, it may be possible to exploit the molecular transport activities of caveolin/caveolae to further investigate the role of caveolin-1 in drug resistance, and potentially develop caveolin-1-based therapeutics.

4.3 Cell-cell/cell-matrix interaction targets

The cell-cell and cell-matrix interactions mediated by caveolin also represent potential therapeutic targets. However, because of limited information at this time it is difficult to identify either general or specific points of intervention. It has been demonstrated previously that a uPAR-integrin-caveolin complex appears to mediate cell-matrix interactions that affect adhesion and motility functions in fibroblasts [27]. Although these activities may be cell type- and matrix-specific,

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one could speculate that antibodies to specific epitopes and/or small molecules that would block such interactions may have therapeutic potential in suppressing relevant cell-matrix attachments and/or motility functions of malignant cells. However, as with other approaches of this type, it will be challenging to build specificity into this system such that high levels of activity will be achieved without limiting toxicities.

5. Molecular surrogates of caveolin-1-based therapeutic targets

The complexity and lack of specific information regarding the exploitation of caveolin/caveolae as a therapeutic target leads to the consideration of surrogate molecular targets with similar properties to those now associated with caveolin-1. Two specific molecular analogies can serve as a foundation for the conceptual development of the caveolin-1 molecule as a model drug target. These examples are presented and discussed in Sections 5.1 and 5.2.

5.1 P-glycoprotein as a drug target for modulatory molecules

The relatively well-defined cholesterol transport functions of caveolin-1/caveolae can be compared to the well-established and extensively studied drug transporter properties of P-glycoprotein [45]. Overexpression of P-glycoprotein leads to drug resistance in part through stimulation of the efflux of multiple structurally unrelated cytotoxic drugs now in clinical use. The concept of modulating P-glycoprotein and potentially other drug resistance genes of this general class of molecules, has been suggested and exploited in the design and development of multiple modulators of P-glycoprotein including Ca^{2+} channel blockers, immunosuppressive agents (cyclosporin A and FK506), analogues of antihypertensive drugs (reserpine and yohimbine), neuroleptic drugs and anti-oestrogens [43]. More recently, the successful modulation of a variety of cytotoxic agents through direct interaction of P-glycoprotein with a small molecule has been reported [49]. This study demonstrates that P-glycoprotein modulation can result in more potent and efficacious cytotoxic activity compared to the cytotoxic agent alone without unwanted pharmacokinetic interactions [49].

It is possible to consider caveolin-1 as a similar target for modulation by small molecules. As with modulators of P-glycoproteins, it would be possible to devise specific screens for small molecules that modulate calveolin-1 functions. Initial screens, and more specific tests of efficacy for selected molecules, could be based on the molecular transport or anti-apoptotic activities of caveolin-1. Certainly, unique considerations must be made for caveolin-1/caveolae in the design and implementation of such a drug screen and include the multiple pleiotropic pathways through which caveolin/caveolae function. However, many of the concepts used for the identification and characterisation of P-glycoprotein modulators can serve as a conceptual/logistical foundation for the development of modulating agents for caveolin-1.

5.2 Anti-apoptotic *bcl-2* family genes

To provide another molecular surrogate for discussion and to potentially reconcile the survival and growth suppressive functions of caveolin-1, it is interesting to consider the well-established properties of *bcl-2* family genes juxtaposed to those of caveolin/caveolae. Interestingly, BCL-2 and other family members can block entry into the cell cycle and thus inhibit growth, as well as provide well-defined protective functions. This property of BCL-2 may be related to its role during normal differentiation as defined in cells such as myelomonocytic progenitor cells. During differentiation, BCL-2 appears to potentiate cell cycle arrest and irreversible withdrawal into the non-proliferating (G_0) state [50]. Subsequent activities of differentiation inducers then allow for the cell to commit to the differentiation pathway. Functional maturation of these cells appears to ultimately downregulate *bcl-2* levels, and thus lead to apoptosis. However, during the maturation process overexpression of *bcl-2* can clearly induce protection from various apoptotic stimuli [50]. Although analogies to human prostatic cell differentiation are certainly tenuous at this point, the fully differentiated and secretory prostatic cell demonstrates low to undetectable levels of caveolin-1 [5] and, therefore, may be poised to undergo apoptosis following a variety of stimuli, including withdrawal of androgenic steroids. Since BCL-2 has also been shown to protect against cytotoxic drugs, it has been suggested that the properties of BCL-2 that are involved in retarding entry into the cell cycle, as well as its protective activities, could provide double protection against these drugs, as both cell cycle entry and response to apoptotic stimuli are required for effective induction of apoptosis by chemotherapeutic agents [51].

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Bcl-2 gene activities have also been considered as a direct target mainly through the use of antisense technologies. One Phase I clinical trial is in progress for prostate cancer at the Memorial Sloan-Kettering Cancer Center with a *bcl-2* antisense oligonucleotide [52]. Antisense technologies represent a viable approach toward reducing caveolin-1 levels *in vivo*, and thus suppressing caveolin functions with regard to metastatic and survival activities. As with all antisense technologies, non-specific interactions of antisense nucleic acids make it difficult to generate a high level of efficacy, and even more difficult to understand and exploit the mechanism of action. However, as with other proteins identified as drug targets, this approach can and should be pursued.

6. Summary

Recent information indicates that caveolin-1 overexpression is associated with metastasis and the androgen-resistant phenotype in prostate cancer [5,34]. Additional reports have also indicated that caveolin-1 overexpression occurs during the development of resistance to chemotherapeutic drugs in multiple human cancer cell lines [35,36]. Co-selection for metastasis and androgen resistance in prostate cancer cells can be conceptually reconciled through consideration of the relevant characteristics of the metastatic environment which, in general, overlap those induced in prostate by castration. Extrapolation of this overlapping selection criteria concept for metastasis/androgen resistance to drug resistance is possible through consideration of the general properties of caveolin/caveolae with regard to cell type-specific anti-apoptotic functions and antiproliferative activities. We have outlined the fundamental molecular pathways through which caveolin/caveolae may regulate multiple cellular and biochemical activities that appear to be responsible, in part, for malignant progression of prostate cancer and potentially other malignancies.

Caveolin-1 and caveolae represent a novel paradigm in drug targeting as caveolae represent multifunctional 'organelles' that are likely established in some malignant cells *de novo* as a consequence of genetic alterations which stimulate caveolin-1 expression. The presence of increased caveolin/caveolae in the cell may than lead to metastatic progression through enhanced cell survival and potentially other altered properties (**Figure 1**). The possible impact of caveolin-1 overexpression on signal transduction pathways, molecular transport and cell-cell/cell-matrix interactions establishes a general foundation for approaching caveolin-1 as a drug target.

At this stage, certain analogies to approaches that led to the modulation of P-glycoprotein, as well as the targeting of BCL-2 family members through antisense technologies, are possible, and can serve as a starting point for the design of specific approaches used in the development of anticaveolin-1 therapeutic agents. As with other molecular drug targets, issues regarding toxicity and specificity are serious concerns and, in the case of caveolae, warrant extreme consideration. Caveolin-1 is expressed in numerous normal cells and, therefore, strategies to specifically target caveolae in specific cell types with anticaveolae modulators or other anticaveolin-1 drugs are of paramount consideration. Although it is relatively early, the emerging role of caveolin-1 as a metastasis-related, androgen resistance and drug resistance gene certainly and rightfully leads to obvious consideration of blocking specific caveolin functions in an effort to develop more effective therapies for metastatic prostate cancers and potentially other malignancies.

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Secreted Caveolin-1 Stimulates Cell Survival/Clonal Growth and Contributes to Metastasis in Androgen-insensitive Prostate Cancer[1]

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Abstract

Caveolin-1 is an integral protein of caveolae, known to play important roles in signal transduction and lipid transport. We demonstrate that caveolin-1 expression is significantly increased in primary and metastatic human prostate cancer after androgen ablation therapy. We also show that caveolin-1 is secreted by androgen-insensitive prostate cancer cells, and that this secretion is regulated by steroid hormones. Significantly caveolin-1 was detected in the high-density lipoprotein L_3 fraction of serum specimens from patients with advanced prostate cancer and to a lesser extent in normal subjects. Conditioned media from high passage caveolin-1 secreting, androgen-insensitive, LNCaP cells stimulated increased viability and clonal growth of low passage, caveolin-1-negative, androgen-sensitive, LNCaP cells *in vitro*, and this effect was blocked by treating the media with caveolin-1 antibody. i.p. injections of caveolin-1 antibody suppressed the orthotopic growth and spontaneous metastasis of highly metastatic, androgen-insensitive caveolin-1-secreting mouse prostate cancer. Overall, our results establish caveolin-1 as an autocrine/paracrine factor that is associated with androgen-insensitive prostate cancer, and we demonstrate the potential for caveolin-1 as a therapeutic target for this important malignancy.

Introduction

Cavelins (designated cav-1, cav-2, and cav-3) are major structural proteins of caveolae, specialized plasma membrane invaginations that are abundant in smooth muscle cells, adipocytes, and endothelium. Numerous studies have shown that cav-1 regulates multiple signal transduction pathways and mediates intracellular trafficking of cholesterol (1–3). We have shown previously that cav-1 mRNA and protein expression are elevated in metastatic mouse and human prostate cancer cells compared with their matched primary tumor counterparts, and that cav-1 expression has independent prognostic value in prostate cancer patients after radical prostatectomy (4, 5). In addition, we demonstrated that enforced cav-1 expression can suppress apoptosis stimulated by withdrawal of growth factors and testosterone or by c-myc overexpression in metastatic prostate cancer cells (6, 7). Interestingly, Liu *et al.* (8) have recently reported that cav-1 is secreted from normal pancreatic acinar cells in response to specific secretagogues such as secretin, cholecystokinin, and Dex.[3] raising the possibility of an autocrine/paracrine or endocrine function for cav-1.

To analyze the expression of cav-1 in androgen-insensitive prostate cancer we determined the pattern of expression for cav-1 in relevant human prostate cancer tissues. We also investigated the possibility that cav-1 is secreted by prostate cancer cells and that secreted cav-1 could influence metastatic progression.

Our results establish cav-1 as an autocrine/paracrine factor that is highly expressed in androgen-insensitive prostate cancer, and we demonstrate the potential for cav-1 as a therapeutic target for this disease.

Materials and Methods

Patients and Specimens. 61 Stage D prostate cancer patients were included in this study. For each patient, one primary prostate cancer and one or more metastatic cancer specimens from different organs were obtained either at the time of radical prostatectomy or at autopsy. From the 11 hormone-refractory patients, a total 33 metastases were derived from lymph node (n = 12), lung (n = 8), bone (n = 1), liver (n = 5), adrenal gland (n = 1), bladder (n = 2), brain (n = 1), and soft tissue (n = 3). Fifty-five metastases were obtained from the 50 nontreated patients, which included lymph node (n = 48), lung (n = 2), bone (n = 2), liver (n = 1), bladder (n = 1), and soft tissue (n = 1). Tissues were fixed in 10% formalin and embedded in paraffin after a routine procedure. Six-μm sections were made from the tissue blocks, and some were stained with H & E for morphological evaluation. They were immunostained using a polyclonal cav-1 antibody and the ABC procedure previously described (4). The immunostained sections were evaluated at a power of ×200 under a Zeiss microscope. For each specimen, the whole cancer area was scanned. Positive cav-1 staining was defined as the presence of any microscopic field in which cancer cells gave rise to cav-1-positive granular immunoreaction products in their cytoplasm. Serum samples were obtained from five patients with radiorecurrent prostate cancer and five healthy individuals. Serum lipoproteins were separated into VLDL, IDL, LDL, HDL_2, HDL_3, and lipid-free protein fractions by KBr density gradient ultracentrifugation after a modified method of Redgrave *et al.* (9).

Cell Culture. Mouse prostate cell lines derived from primary tumors (148-1PA and 151-1PA) or metastatic deposits (178-2BMA, 148-1LMD, 151-2LMB, 151-2LMC, and 151-1LM1), were cultured as described previously (6, 10). The growth media for the different cells was in 10% fetal bovine serum as follows: RPMI 1640 for LNCaP; MEM-NEAA for DU145; F12K for PC3; F12K supplemented with heparin and endothelial cell growth supplement for human umbilical vascular endothelial cell; and DMEM for human intestinal smooth muscle and all mouse cells. The human cav-1 cDNA in pcDNA3.1 was transfected into LNCaP cells with Tfx reagent (Promega, Madison, Wisconsin; Ref. 7). For conditioned medium preparation, subconfluent cultures were washed three times with PBS and incubated with SFM for 24 h; the media was collected and contamination of membranous cav-1 from cell debris was minimized by centrifugation at 1,000 × g and then at 100,000 × g. Conditioned

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[3] The abbreviations used are: Dex, dexamethasone; HDL, high-density lipoprotein; TCA, trichloroacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LP-LNCaP, low-passage LNCaP cells; HP-LNCaP, high-passage LNCaP cells; DHT, dihydrotestosterone; SFM, serum-free medium; Tg, thapsigargin.

media for *in vitro* viability assays (see below) was concentrated ×20 and treated with cav-1 antibody or IgG (10 μg/ml) and incubated for 4 h at 4°C.

Western Blot Analysis. Conditioned media collected and centrifuged as described above and 1 ml concentrated by TCA precipitation. The precipitate was redissolved in 70 μl of SDS sample buffer and 30 μl were loaded per well. Proteins obtained from lysed cells and from TCA-precipitated conditioned media were separated by 12% SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were prestained with Ponceau S before blocking to verify even loading. The membranes were blotted with rabbit polyclonal cav-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 24 h at 4°C, with shaking. After incubation with horseradish peroxidase-conjugated secondary antibodies (ICN/CAPPEL, Aurora, OH), the binding was detected by enhanced chemiluminescence, Super Signal (Pierce Chemical Co., Rockford, IL).

Cell Viability Assays. The viability by MTT was measured according to the previously described method (6). The cell viability was also measured after the initial period of incubation by an ATP-based assay using the Packard ATPLite-M kit (Packard, Meridian, CT) according to the manufacturer's procedure, and the cell viability was expressed as the amount of light production/cell number in each well. For the clonogenic assay, 200 cells were treated in SFM or conditioned media as described in the cell viability assay. The medium was then carefully removed, and cells were trypsinized and reseeded to 10-cm plates with complete medium and grown for 10–15 days. Colonies were stained with MTT in the culture medium, and the numbers of colonies were counted using Advanced Colony Counting software (NucleoTech Corp., Hayward, CA). Each experiment was repeated three to five times.

***In Vivo* Metastasis Analysis.** Orthotopic tumors of 178–2BMA in male mice were generated by injecting 5000 cells directly into the dorso-lateral prostate. The mice were subsequently treated with control rabbit IgG or rabbit polyclonal cav-1 antibody via i.p. injections (10 μg/animal ~~twice a~~ day). After 3 weeks, the tumors were removed and the wet-weight determined. Metastatic activity to lung was evaluated by counting Bouin's fixative-stained lungs under a dissecting microscope. Femur bones and pelvic lymph nodes were removed and processed for paraffin sections after a routine procedure. Five-μm sections were cut and stained with H&E for morphological evaluation. To better identify metastatic cancer cells in bone marrow, the adjacent sections were immunostained using the standard ABC procedures (MOM kit; Vector Laboratories, Burlington, CA) in conjunction with a monoclonal antibody to cytokeratin 8 (Dako Corp., Carpinteria, CA). The metastatic cancer cells were counted and data expressed as cancer cell number/microscopic field of bone marrow area. Pelvic lymph nodes metastatic cancer cells were labeled with cytokeratin-18 antibody. The incidence of positive lymph nodes was recorded for each animal and the percentages of metastatic cancer deposits in individual lymph nodes were also measured.

Results and Discussion

We evaluated the effect of androgen ablation therapy on cav-1 expression by immunohistochemistry in a large panel of primary tumor tissues and specimens of lymph node, bone, and soft tissue metastases from stage D prostate cancer patients obtained before and after hormonal treatment (Table 1). Previous studies using a distinct set of tissue specimens showed that the frequency of cav-1 positivity was 8% in normal prostatic epithelia; 29% in primary cancers with nodal metastases; and 33% in nodal metastases *per se* (4). In this study, the frequency of cav-1-positive primary prostate cancers was increased from 38% in hormonally naive patient group to 73% in the hormone refractory patient group ($P < 0.05$; χ^2 test). Cav-1 positivity was demonstrated in 82% of the metastatic specimens from patients who had not been treated with hormone therapy, and this frequency was also significantly increased to 82% of metastases from patients treated with hormones ($P < 0.05$; Mann-Whitney test).

Additional analysis demonstrated that the percentage of cav-1-positive cells was significantly increased from 18.6% in primary tumors to 29.9% in hormone-treated primary tumors ($P < 0.05$; Mann-Whitney test). The percentage of cav-1-positive cells in metastatic specimens of treated patients was higher (38%) than in specimens from untreated patients (35.5%), but this increase was not significant ($P > 0.05$; Mann-Whitney test). Increased cav-1 positivity in hormone-refractory prostate cancer is consistent with several reports that have correlated overexpression of cav-1 with multidrug resistance independent of P-glycoprotein in human cancer cell lines from various tumor types (11–13). It was of interest that the highest percentage of cav-1-positive cells documented (hormone refractory metastases) approached but did not exceed 40%. These results prompted us to investigate the possibility that cav-1 is secreted by prostate cancer cells and functions as a paracrine/autocrine factor.

Cav-1 was detected in conditioned media from androgen-insensitive mouse (148-1PA, 151-1LM1, 178-2BMA, 148-1ABAC3, 151-1PA, 151-2LMC, 151-2LMB, and 148-1LMD), and human (DU145, PC3, and TSU-Pr1) prostate cancer cells in variable amounts. In androgen-sensitive, LP-LNCaP cells, cav-1 was not expressed; yet in HP-LNCaP cells that had reduced androgen-sensitivity, cav-1 was expressed and secreted into the medium. In contrast, nonprostatic cells such as endothelial, fibroblast, and smooth muscle had a substantial amount of intracellular cav-1 yet minimal or nondetectable levels of cav-1 in their conditioned media (Fig. 1*A*).

We used the mouse prostate cancer cell line 178-2BMA, derived from a bone metastasis generated from the metastatic mouse prostate reconstitution model, (10) and HP-LNCaP to test the possible regulation of cav-1 secretion by DHT and Dex *in vitro*. Both cell lines were shown to be insensitive to androgen *in vitro*, *i.e.*, no significant changes in cell number or viability were detected under serum-free conditions in the presence and absence of 10 nM testosterone (data not shown). The results showed that cav-1 (M_r 21,000) was secreted by 178-2BMA cells in response to these steroid hormones, reaching the highest levels at 10 nM DHT and 100 nM Dex (Fig. 1*B*). The increase in secreted cav-1 in response to these secretagogues was paralleled by a decrease in intracellular cav-1 (not shown). A similar pattern for cav-1 secretion was observed in HP-LNCaP cells (Fig. 1*B*).

We investigated the secretory route for cav-1 by expressing human cav-1 in cav-1-negative LP-LNCaP cells. After transfection, a substantial amount of ectopically expressed cav-1 was detected in the media compared with that in the cell lysate, and cav-1 secretion was increased in response to DHT. Cav-1 was not detected in the media or cell lysate of the vector control-transfected cells, yet all transfected cells excreted prostate-specific antigen into the media in a DHT-regulated fashion (Fig. 1*C*). These results show that cav-1 is secreted by androgen-insensitive mouse and human prostate cancer cells in response to specific steroid hormones. Although we do not provide evidence for the mechanism by which cav-1 enters the secretory

Table 1 Cav-1 expression in Stage D prostate cancers in response to hormonal treatment

Hormone treatment	Frequency of cav-1-positive cancers [% (*n*)]		Percentage of cav-1-positive cancer cells [mean (± SE)]	
	Primary	Metastases	Primary	Metastases
No	38 (19/50)	62 (34/55)	18.6 (2.4)	35.5 (6.0)
Yes	73 (8/11)	82 (27/33)[a]	29.9 (5.9)[b]	38.1 (4.8)

[a] $P < 0.05$ (χ^2 test).
[b] $P < 0.05$ (Mann-Whitney test).

pathway, the results show that ectopically expressed cav-1 is secreted by LNCaP cells, and that secreted cav-1 migrates on SDS-PAGE similarly to that derived from endothelial cells and fibroblasts, suggesting that the secreted form is not modified posttranscriptionally. To determine whether cav-1 could also be secreted by human prostate cancer cells *in vivo*, we fractionated human serum and analyzed various fractions for cav-1. Our results revealed that cav-1 is specifically detected in the serum HDL_3 lipoprotein subfraction, and that cav-1 levels may be higher in the serum of prostate cancer patients compared with the serum of normal individuals (Fig. 1*D*).

The function of secreted cav-1 was investigated by testing the effects of concentrated conditioned media collected from HP-LNCaP cells on LP-LNCaP cell viability and clonal growth under serum-free conditions. The results indicate that secreted cav-1 was capable of promoting viability, using a standard MTT method (Ref. 6; Fig. 2*A*) or luminescent technique (Packard ATPLite; Fig. 2*B*) and of stimulating viability/clonal growth using a clonogenic assay (Fig. 2*C*). To test whether such activities would be specific for the cav-1 molecule, polyclonal cav-1 antibody was added to conditioned media or rabbit IgG as a control. Treatment of the conditioned media with anti-cav-1 antibody reduced the viability significantly ($P < 0.001$ for MTT and clonogenic assays and $P < 0.0001$ for ATPLite assay) compared with the IgG-treated medium. We also tested the effect of secreted cav-1 on Tg-induced apoptosis in LP-LNCaP cells. Tg promotes apoptosis (14), characterized by caspase activation and the appearance of apoptotic bodies in these cells (data not shown). The results indicated that secreted cav-1 was able to protect the cells from the apoptotic effects of this drug (Fig. 2*D*). These studies revealed that media containing secreted cav-1 generates antiapoptotic activities in prostate cancer cells similar to those elicited after enforced expression of cav-1 within the cell (6, 7).

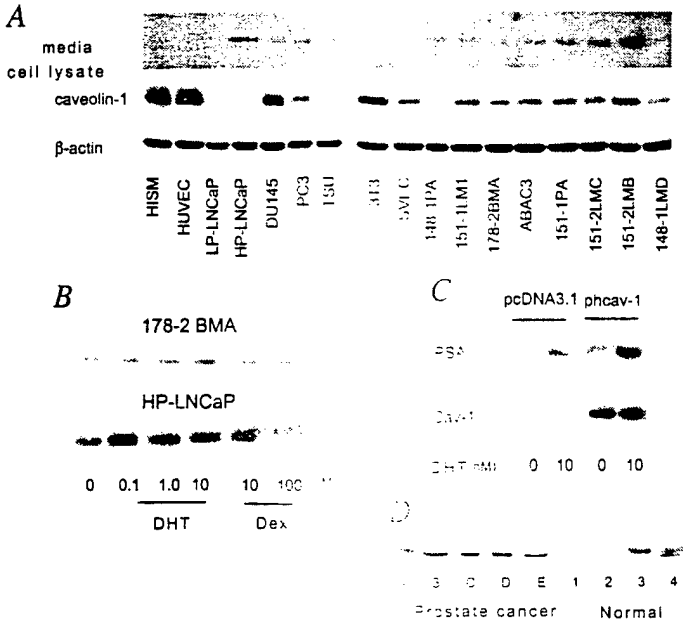

Fig. 1. Cav-1 secretion assessed by Western blot analysis. *A*, Cav-1 in human and mouse prostate cancer cell lysates and conditioned media. The protein concentration in each well was controlled by loading equal quantities of cell lysate and equal volumes of TCA-precipitated conditioned media. Loading was monitored by staining the membrane after transfer with Ponceau S. No visible protein bands were detected in the conditioned media lanes. *B*, Cav-1 secretion by HP-LNCaP and 178-2BMA cells in response to DHT and Dex. Conditioned media were collected after incubation of subconfluent cells with SFM in the presence or absence of hormones for 24 h. *C*, effect of DHT on the ectopically expressed cav-1 secretion in LP-LNCaP cells compared with that of endogenous prostate-specific antigen. Cells were transfected with cDNA encoding for full-length human cav-1; 48 h after transfection, cells were incubated in SFM with or without DHT for 24 h; 0.5 ml of TCA-precipitated media was analyzed. *D*, cav-1 in dialyzed and lyophilized serum HDL_3 fraction (50 μg of protein) of prostate cancer patients and healthy individuals.

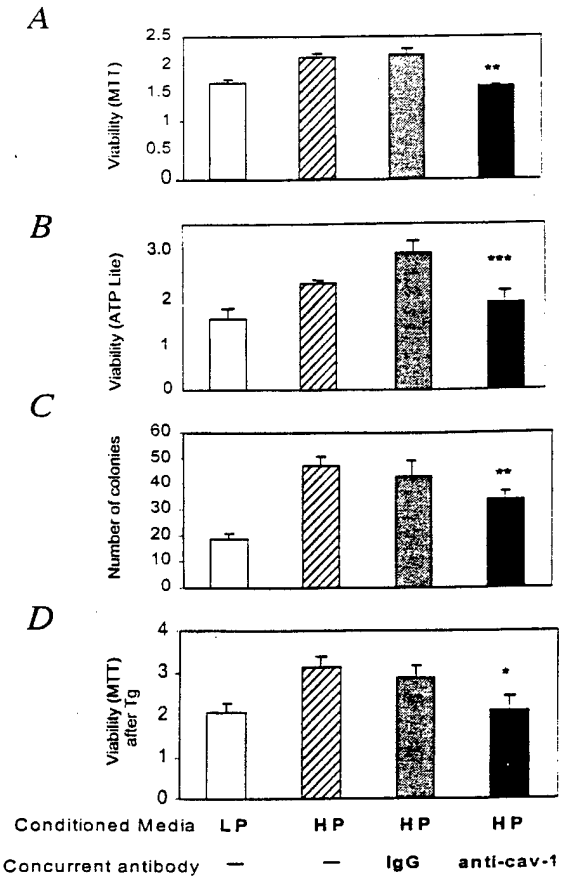

Fig. 2. Effect of cav-1 in conditioned media on LP-LNCaP cells. *A*, cell viability measured by MTT assay. Cells were incubated in SFM as a control or in conditioned media for 72 h and stained with MTT, and viable and dead cells were counted. The viability is expressed as a fold of increase relative to SFM. *B*, ATPLite assay for cell viability on cells treated as in *A*. *C*, clonogenic assay for the effect of secreted cav-1 on LNCaP cells. Cells were treated for 72 h in conditioned media then transferred to growth media. *D*, effect of secreted cav-1 on the viability of LNCaP cells treated with Tg. cav-1 protected the cells from the Tg (0.2 μM) -induced apoptosis. *, $P < 0.05$; **, $P < 0.001$.; ***, $P < 0.0001$ (ANOVA).

We then tested whether blocking secreted cav-1 activity *in vivo* with specific antibodies would result in therapeutic activity, potentially through abrogation of the antiapoptotic effects of secreted cav-1. Androgen-insensitive 178-2BMA cells that spontaneously metastasize with high frequency (nearly 100%) to lung, lymph nodes, and bone were grown as orthotopic tumors in adult male mice. After 21 days of treatment with cav-1 antibody or IgG, the animals were killed. The mean tumor wet-weight (Fig. 3*A*) and the mean number of lung metastases (Fig. 3*B*) of the anti-cav-1-treated group was significantly lower than the IgG-treated group ($P < 0.01$ and $P < 0.05$, respectively). The cav-1 antibody-treated group also had a significantly lower percentage of cancer cell volume in lymph nodes ($P < 0.01$; Fig. 3*C*). The metastatic cell density in the bone marrow (Fig. 3*D*) was also reduced significantly ($P < 0.05$) in the cav-1 antibody-treated mice compared with those of the IgG-treated group. These results show that neutralization of secreted cav-1 *in vivo* by specific antibody suppresses primary prostate tumor growth and spontaneous metastasis to the lung, lymph nodes, and bone.

Overall, the results of this study contribute significantly to the understanding of androgen-insensitive prostate cancer. Previous studies have documented that bcl-2 overexpression may characterize a subset of androgen-insensitive disease (15). The aberrant expression of HER-2/neu has been implicated in androgen independence in animal models (16) and by immunohistochemical analyses of human specimens (17). However, as reviewed by Scher (18), the role of

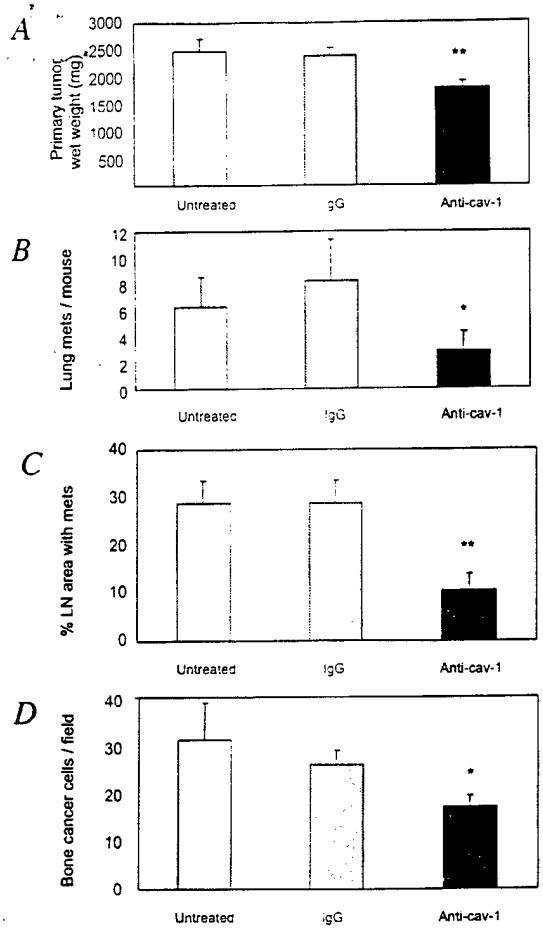

Fig. 3. Antitumor and antimetastatic effect of cav-1 antibody treatment in mice with 178-2BMA orthotopic tumors. *A*, prostate tumor wet-weight in untreated, IgG-treated, and cav-1 antibody-treated mice. *B*, number of lung metastatic cells. *C*, percentage of volume of cancer deposits in lymph nodes. *D*, bone marrow metastatic cell density. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test).

HER-2/neu in prostate cancer progression is not as self-evident as it is in breast cancer. In this study, we demonstrate that cav-1 up-regulation is associated with the development of androgen-insensitive prostate cancer, and that androgen-insensitive prostate cancer cells secrete biologically active cav-1 in a steroid-regulated fashion. We have shown that testosterone up-regulates cav-1 expression in prostate cancer cells, in part, through transcriptional activation.[4] Therefore, in the presence of testosterone, cav-1 expression and/or secretion may be significantly stimulated in prostate cancer cells. Androgen ablation may select for alternative pathways of cav-1 regulation that could include glucocorticoid-stimulated cav-1 secretion, as shown here. It was shown previously that polypeptide growth factors can regulate cav-1 expression in NIH 3T3 cells (19). Therefore cav-1 expression and secretion may be stimulated initially by androgens, yet subsequent androgen ablation may select for alternative pathways that sustain cav-1 activities and thus transition the malignant cell into an androgen-insensitive phenotype. As shown in this study, secreted cav-1 can stimulate viability and clonal growth in adjacent prostate cancer cells that do not express cav-1.

The concept of a secreted autocrine/paracrine factor that directly contributes to androgen resistance in prostate cancer is novel and represents an efficient mechanism for maximizing resistance to various proapoptotic stimuli that metastatic cells often encounter during the highly inefficient process of metastasis (20). Our *in vivo* studies indicating that cav-1-specific antibody delivered i.p. can suppress malignant progression of androgen-insensitive, cav-1-secreting mouse prostate cancer cells are remarkable. These results not only indicate that secreted cav-1 promotes metastasis *in vivo*, but also raise the possibility of using cav-1 as a therapeutic target for androgen-insensitive disease. It is conceivable that when combined with anti-androgen therapy or potentially with chemotherapy, cav-1-specific antibody therapy may have greater therapeutic activity. Additional studies will be required to address this issue.

[4] Li *et al.*, manuscript submitted.

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CAVEOLIN-1 MEDIATES TESTOSTERONE-STIMULATED SURVIVAL/CLONAL GROWTH AND PROMOTES METASTATIC ACTIVITIES IN PROSTATE CANCER CELLS

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The abbreviations used are: T, testosterone; cav-1, caveolin-1; SFM, serum free medium; AR, androgen receptor.

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ABSTRACT

Previously we demonstrated that upregulation of caveolin-1 (cav-1) was associated with prostate cancer metastasis, biochemical recurrence following radical prostatectomy and androgen insensitivity. The objective of this study was to characterize the regulation of cav-1 by testosterone and to test the effects of cav-1 on prostate cancer cell survival/clonal growth and metastatic activities. Our results demonstrated that testosterone upregulated cav-1 protein levels in part through transcriptional regulation and significantly enhanced survival of prostate cancer cell lines, ABAC3 and LNCaP, following serum starvation (>40% and >60% increased viability, respectively) and in an extended clonogenic assay (approximately 4-fold and 6-fold increased colonies, respectively). Importantly, antisense cav-1 inhibited the survival effects of testosterone in these assay systems. Modest but not high levels of adenoviral vector-mediated cav-1 expression alone also significantly increased viability (>40%) and clonal growth (10-fold increased colonies) after serum starvation. Analysis of spontaneous metastasis in stably transfected antisense cav-1 mouse prostate cancer cell clones demonstrated reduction of spontaneous lymph node metastasis incidence (13%), spontaneous lymph node metastasis volume (46%), and experimental lung metastasis incidence (40%) compared to vector control cell clones. Surgical castration further reduced spontaneous lymph node metastasis incidence and volume (18% and 28% respectively) in antisense cancer cell clones but not in vector control clones. Our studies demonstrate that cav-1 is a downstream effector of testosterone mediated prostate cancer cell survival/clonal growth and that modest levels of cav-1 can independently promote prostate cancer cell survival/clonal growth and metastatic activities.

INTRODUCTION

Prostate cancer is a continued threat to the lives of tens of thousands of US men, despite efforts to control this disease through screening of asymptomatic men and the aggressive use of surgery and irradiation therapy for presumed localized disease (1). Unfortunately, many men continue to present with advanced prostate cancer or recur from localized therapy, and there are no curative therapies available for androgen resistant metastatic disease. Although prostate cancer was shown to be initially responsive to androgen ablation more than fifty years ago (2), there is only minimal understanding at the mechanistic level regarding the ultimate hormone resistant state of prostate cancer that is responsible for the exceedingly high mortality rate. Previously we reported that caveolin-1 (cav-1) levels were elevated in metastatic mouse and human prostate cancer (3) and that cav-1 positivity had independent prognostic value for cancer recurrence following radical prostatectomy (4). Additional studies demonstrated that suppression of cav-1 levels led to re-establishment of androgen sensitivity *in vitro* and *in vivo* and that enforced cav-1 expression could convert androgen sensitive prostate cancer cells to androgen insensitive cells (5). Other reports have shown that cav-1 is upregulated in multidrug resistant cancer cells and in some cases this upregulation is independent of P-glycoprotein (6-8). More recently cav-1 was shown to suppress c-myc-induced apoptosis in Rat1A and LNCaP cells (9).

Cav-1 is the principal component of caveolae, sub-invaginations of the plasma membrane and trans-Golgi network that have been implicated in sphingolipid-cholesterol transport and signal transduction pathways (reviewed in 10-13). Under some conditions cav-1 has been shown to suppress growth of specific cell lines *in vitro* and *in vivo* (8,14-17), and it has been suggested that cav-1 functions as a tumor suppressor gene (18). However, specific genetic analysis of cav-1 did not support this contention (19, Ren *et al.*, unpublished data). Recent studies have indicated that some genes can manifest seemingly opposing functional activities in a context dependent fashion. One example is the bcl-2 gene that can demonstrate pro- or anti-apoptotic activities depending upon its level of expression (20). These opposing functions may be related to separate bcl-2 protein domains that have been shown to independently mediate growth arrest or survival depending on cell context (21,22). Additional examples

are the Cox-1 and Cox-2 genes that have been shown to be upregulated in numerous human malignancies, yet overexpression of these genes can suppress growth and induce apoptosis *in vitro* (23,24). Recent studies suggest that the growth suppressive effects of Cox-1 are not related to its enzymatic activities within the prostaglandin synthesis pathway (24). Overall, these results indicate the need to clearly define the regulation, biological activities and mechanism(s) of action for these multi-potential genes within the context of malignant progression. In this report, we demonstrate that cav-1 is a downstream effector of testosterone (T)-mediated survival activities and that modest but not high levels of cav-1 can promote both cell survival and metastatic activities in mouse and human prostate cancer cells.

MATERIALS AND METHODS

Cell lines and cell culture. The various metastatic mouse prostate cancer cell lines were generated from tumors initiated by retroviral transduction of the *ras* and *myc* oncogenes into fetal prostate tissues from p53 homozygous mutants using the mouse prostate reconstitution (MPR) model (25). The 148-1PA cell line was established from a primary carcinoma and 148-1LMD was established from a lung metastasis from the same mouse. The ABAC3 and ABAC5 clonal cell lines were derived from 148-1LMD by introduction of an antisense mouse cav-1 cDNA as previously described (5). Similarly, antisense clone BACS4 was derived from 151-2LMC, a lung metastatic clone from a different mouse (5,25). ABH11, ABH14 and BHS3 are empty vector clones derived from either 148-1LMD or 151-2LMC and used for controls (5). The mouse cell lines were grown in DMEM with 10% FCS. The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection, grown in RPMI-1640 with 10% FCS, and used at passage 30-60. The appropriate media without serum but with 0.1% bovine serum albumin was used as serum free medium (SFM). All cells were routinely grown at 37 ^{o}C with 5 % CO_2.

MTT assay. Subconfluent cells were trypsinized, collected by centrifugation and washed once with SFM. A single-cell suspension was then seeded at low cell density (~200 cells per well of a 96-well

plate) in SFM, or SFM with testosterone (Sigma, St Louis, MO) (SFMT). After 3 days, viability of cells was determined by incubation with 0.05 mg/ml of MTT (Sigma) at 37 °C for two hours to overnight. The viability (viable cells/total cells) was determined by counting blue (viable) and total cells microscopically and was expressed as relative cell viability by normalization to control (control = 1; in cav-1 induction experiments, T = 0 nM was used as control; in virus infection experiments, uninfected was used as control). Previous experiments demonstrated that under conditions of growth/survival depletion and low cell density, proliferation was minimal and therefore the activities monitored by this MTT assay represents predominately cell viability (5). The viability data are representative of at least three independent triplicate experiments. Error bars show standard deviations of a triplicate experiment.

ATPLite assay. ABAC3 (1 x 10^4 cell/well) and LNCaP (1 x 10^5 cells/well) cells were seeded in SFM or in SFM with various concentration of T in 12-well plates. After 3-days, floating cells and trypsin-detached cells were combined and counted with a Coulter Particle Counter (Coulter Corp., Miami, FL). One thousand cells were seeded into each well of 96-well black culture plate (Packard Instrument Co, Meriden, CT). Cell viability was determined with a luminescent ATP detection kit, Packard ATPLite-M according to manufacturer's directions. Light units generated by ATP in each sample were normalized to control (T= 0 nM) and expressed as relative ATP level. The ATPLite assay was also performed on LNCaP cells infected with an adenoviral vector expressing human sense cav-1 or with control RSV adenoviral vector in 6-well plates as described below. After 2 days in the complete medium post infection, cells were subjected to growth/survival factor depletion for 3 days in SFM then collected for ATP determination.

Clonogenic assay. Cells were suspended at low density in SFM or SFMT (T= 10 nM for ABAC3 and 5 nM for LNCaP) in 96-well plates as described for MTT assay. After 3 days, the medium was carefully removed, cells trypsinized and re-seeded in 10-cm plates at a density of 10^3 cells/plate with complete medium. After 10-15 days colonies were stained with 0.05 mg/ml of MTT in the culture

medium for 30 minutes and the number of colonies counted using Advanced Colony Counting software after capturing the image of each plate with a NucleoVision image analysis system (NucleoTech, Hayward, CA). Adenoviral vector infected cells were grown in complete medium for 2 days after infection and then subjected to low cell density growth/survival factor depletion for 3 days. Cells were then seeded into 10-cm plates at a density of 10^3 cells/plate in complete medium. Colonies counted as above after 3 weeks.

Induction of cav-1 protein by T. Cells were seeded at a density similar to that used in the viability assay (2.0×10^5 cells/15-cm plate) in SFM or SFMT with varying concentrations of T. After two days cells were scraped from plates and collected by centrifugation. The cell pellets were washed once with PBS and then lysed with TNES lysis buffer (50 mM Tris (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1% NP40, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM PMSF) on ice for 45 minutes. Proteins were separated on a 12% polyacrylamide-SDS gel and then electrophoretically transferred onto a nitrocellulose membrane. Cav-1 and androgen receptor (AR) were detected with purified polyclonal cav-1 antibody (SC894) and polyclonal AR antibody (SC-826) (Santa Cruz Biotech, Santa Cruz, CA). A β-actin monoclonal antibody (A5441, Sigma) was used to detect β-actin for loading control. All western blots shown are representative of at least three independent experiments.

Luciferase assay for the mouse cav-1 promoter reporter. A 721 bp mouse cav-1 promoter sequence was subcloned into the luciferase reporter vector, pGL3-basic (Promega Corp., Madison, WI) to generate a mouse cav-1 promoter controlled luciferase reporter vector, pGL3-mcav-1-luc (9). One μg of pGL3-mcav-1-luc or pGL3-basic was cotransfected with 0.25 μg of pCMV-β-gal into ABAC3 cells (per well of a 6-well plate) using Lipofectamine Plus (Life Technologies, Grand Island, NY) according to manufacturer's protocol. Three hours after lipofection, fresh medium was added and the FCS concentration brought to 10%. Twenty-four hours later, the cells were trypsinized, washed once with

SFM, and a single cell suspension seeded in SFM or SFMT (T= 20 nM) at low density (2 x 10^5 cells/ 10 cm plate). Cells were collected after 24 hours, and lysed in 50 µl of LucLite substrate buffer (Packard) for 15 minutes at room temperature and then diluted to desired volume with PBS containing 1 mM Mg^{2+} and 1 mM Ca^{2+}. Luciferase assays were performed using Packard LucLite kit (Packard) and luciferase activities were measured on a TopCount luminescence counter (Packard). β-galactosidase activity was measured as an internal control for the transfection efficiency using a β-galactosidase assay kit (Promega). Tfx™-50 reagent (Promega) was used for the transfection of LNCaP cells. Two µg of pGL3-mcav-1-luc or its control vector, pGL3-basic, was cotransfected with 0.25 µg of pCMV-β-gal into LNCaP cells using 2:1 charge ratio of Tfx™ reagent to DNA. One hour after transfection, 2 ml of fresh SFM or SFMT were added to each well (final [T]= 10 nM). The androgen antagonist casodex (1 µM) was also added to the selected SFMT wells. Cells were harvested and cell lysates were prepared 48 hours after transfection. The reporter activity was expressed as relative luciferase activity (light units) by normalization to β-galactosidase activity. The data reported are representative of at least three independent experiments.

Adenoviral vector-mediated sense and antisense human cav-1 expression. Recombinant adenoviral vectors containing sense (AdScav-1) or antisense (AdAScav-1) human cav-1 cDNA or control AdRSV without a cDNA were generated as previously described (5,9). LNCaP cells were seeded at a density of 5.0 x 10^5 cells/well in 6-well plates. After incubation overnight the medium was replaced with 1 ml SFM and adenoviral-vector at different multiplicities of infection (MOI) were added. After 3 hours, the medium was removed and replaced with complete culture medium. After 48 hours the cells were trypsinized for MTT assay and for the preparation of protein lysates. For ATPLite and clonogenic assays, the culture medium was replaced with SFM 48 hours after infection and the cells subjected to growth/survival factor depletion for 3 days prior to each assay. Expression of cav-1 was also confirmed in adenoviral vector infected cells by immunostaining with cav-1 antibody as previously described (4).

A double infection with AdAScav-1 was adopted to minimize endogenous cav-1. ABAC3 cells were seeded at 1.0 x 10^5/well of a 6-well plate and grown overnight. The next day (day 1) cells were infected with the adenoviral-vector at the indicated MOI. A second infection was performed on day 3 (MOI calculations adjusted for increased cell number), followed by another two day growth period in complete medium. On day 5, the cells were trypsinized, washed with SFM, and seeded in SFM or SFMT (T= 10 nM) at low cell density as described above for examination of cav-1 protein expression and viability. For the clonogenic assay, SFM or SFMT treated cells were detached from 96-well plate and seeded into 10 cm plates for colony counting as described above.

***In vivo* metastasis analyses.** A panel of mouse stable antisense cav-1 clones (ABAC3, ABAC5, BACS4) and control vector clones (ABH11, ABH14, BHS3) established from high cav-1 expression lung metastatic cell lines, 148-1LMD or 151-2LMC (3) were used for orthotopic injection or tail vein injection into syngeneic 129/SV mice as previously described (26). Each cell clone was injected into eight to nine animals. Orthotopic tumors were established by injection of 5,000 cells into the dorsolateral prostate, a number sufficient to establish a 100% tumor take (26). In some experiments animals were surgically castrated or received sham surgery three days following orthotopic inoculation as previously described (5). Two weeks after orthotopic inoculation animals were euthanized and the tumor carefully excised and the wet weight recorded. The pelvic and retroperitoneal lymph nodes were excised, placed in formalin, paraffin embedded, cut into 4-5 μm sections, and stained with hematoxylin and eosin for histologic examination. The extent of metastasis was assessed quantitatively on the stained slides via computer-assisted image analysis (5). An experimental metastasis assay consisted of the tail vein injection of 50,000 cells. Mice were euthanized after 14 days and the lungs weighed, fixed in Bouin's fixative, and visible lung metastases counted with the aid of a dissecting microscope at 10X magnification.

All mice were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care and all experiments conducted in accordance with the principles and

procedures outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Statistical analysis. Statistical analyses were performed with Statview 5.0 (SAS Institute, Inc, Cary, NC). Significance was determined by analysis of variance with Fisher's PLSD.

RESULTS

T significantly enhances cell survival of androgen-sensitive prostate cancer cells under conditions of growth/survival factor depletion. We initially tested the capacity of T to stimulate viability of both mouse and human prostate cancer cells *in vitro* using both MTT assay and ATPLite assay in SFM with various concentrations of T. As expected, T significantly enhanced cell survival of androgen-sensitive prostate cancer cells following growth/survival factor depletion. In both the MTT assay and the ATPLite assay maximum viability was observed at 10 nM of T for ABAC3 mouse prostate cancer cells with >40% increased viable cells compared to SFM (T = 0 nM) ($P < 0.0001$) (Fig. 1*A&B*). For the human prostate cancer cell line LNCaP, maximum protection was observed at 5 nM of T with >60% ($P < 0.0001$) increased viability (Fig 1*A&B*). To analyze the long-term effects of T stimulated viability, we extended the viability assay to a modified clonogenic assay (see Materials and Methods). The results revealed a long-term T stimulation resulted in ~4-fold increased colonies for ABAC3 and ~6-fold more colonies for LNCaP (Fig. 1*C*).

Dose-dependent upregulation of cav-1 protein by T. To analyze the relationship between T levels and cav-1 expression, we determined the dose-dependent effect of T on cav-1 protein expression in mouse and human prostate cancer cell lines. 148-1PA and ABAC3 cells showed maximal induction of cav-1 protein at 20 nM T (Fig. 2). LNCaP cells demonstrated slightly higher sensitivity to T with

maximal induction of cav-1 at 5-10 nM T (Fig. 2). These results are in agreement with and extend a previous report demonstrating increased cav-1 protein levels following the treatment of T *in vitro* (27).

Transcriptional activation of Cav-1 promoter by T. To determine whether upregulation of cav-1 by T occurs at the level of transcriptional regulation we used a luciferase reporter vector under the transcriptional control of the mouse cav-1 promoter, pGL3-mcav-1-luc. The relative activity of the cav-1 promoter was increased more than 2-fold ($P < 0.0001$) by T in ABAC3 cells and approximately 2-fold ($P < 0.0001$) by T in LNCaP cells (Fig. 3). This activity could be blocked by the addition of 1μM casodex, a direct AR antagonist (Fig. 3), indicating that the upregulation of cav-1 by T is mediated by AR.

Antisense cav-1 significantly inhibits the effects of T on cell survival and clonal growth. Since T enhanced survival and induced cav-1 expression, we asked whether survival activities induced by T were a consequence of the up-regulation of cav-1. To address this we infected ABAC3 cells with AdAScav-1 followed by T stimulation. The results in figure 4*A* show that antisense cav-1 abrogated T stimulated cav-1 up-regulation (compare AS25 to Un or R25 and AS50 to Un or R50). To demonstrate that the suppression of cav-1 up-regulation by T was a specific consequence of direct cav-1 gene antisense suppression rather than the result of a general toxic and/or non-specific effects on cell viability, we also evaluated the expression of a known androgen responsive gene, AR (28). The up-regulation of AR by T was not altered in the antisense cav-1 treated cells confirming the cav-1 specificity of the antisense suppression (Fig. 4A). As a consequence of the suppression of cav-1 upregulation by T, the increased viability stimulated by T was significantly reduced (Fig. 4*B*). At an MOI of 25 with the control AdRSV vector (R25) relative cell viability in SFMT was increased to 1.42 relative to SFM ($P = 0.0007$) whereas with antisense cav-1 (AS25) relative survival was only 1.1 in SFMT ($P = 0.3214$). Similarly, at MOI = 50, T stimulated survival was reduced from 1.5 (R50, $P = 0.0003$) to 1.07 (AS50, $P = 0.5208$). In the clonogenic assays (Fig. 4*C*), T stimulated ~2-3-fold increase colonies in uninfected and RSV controls ($P < 0.0001$), whereas in antisense cav-1 infected, SFMT treated cells the colony number was increased

by 41% in AS25 (P = 0.09954) and 22% in AS50 (P = 0.3256). The results of these experiments together with those described above demonstrate that cav-1 is a downstream effector of T that is responsible, in part, for the survival/clonal growth stimulated by T in prostate cancer cells under these experimental conditions.

Cav-1 expression promotes survival and clonal growth. To determine whether cav-1 can promote survival in human prostate cancer cells in the absence of T, we infected LNCaP cells with AdScav-1. Dose-dependent expression of human cav-1 was achieved with increasing MOI (Fig. 5*A*). Cav-1 expression increased the relative viability of LNCaP cells following growth/survival factor depletion. Interestingly, the maximum survival protection of cav-1 (> 40%, P < 0.0001) was observed at MOI = 10 (Fig. 5*B&C*), which corresponded with a moderate level of cav-1 protein (Fig. 5*A*). Viability protection was observed with AdScav-1 at MOI 5-50, but not MOI = 100 or 200 (Fig. 5*B&C*). These data clearly suggest that cav-1 can induce survival activities when it is expressed at moderate levels, yet when expressed at high levels, cav-1 may be toxic to the cells. To determine the long-term effect of cav-1 expression on survival/clonal growth, we also performed the clonogenic assay on infected cells. A significant difference between the effects of AdScav-1 and control vector AdRSV was observed in this extended assay, with ~8-10-fold more colonies for the cav-1 group in a 3-week period (Fig. 5*D*). To confirm expression of cav-1 in the LNCaP cells we performed immunohistochemical staining which revealed an absence of cav-1 in uninfected LNCaP cells (Fig. 5E) but readily detectable expression in LNCaP cells infected with AdScav-1 (Fig. 5F).

Reduced cav-1 expression in metastatic mouse prostate cancer cells results in suppression of metastasis *in vivo*. To test the effects of cav-1 expression on metastatic activities *in vivo*, we analyzed spontaneous (lymph node metastasis from orthotopic tumors) and experimental (tail vein injected cells) metastasis in a panel of high cav-1 lung metastasis-derived mouse prostate cancer cell lines stably transfected with antisense cav-1 or control vector (5). The growth of the cell lines as orthotopic tumors

was compared to vector controls following sham surgery (Fig. 6A). The antisense clones were about 10% smaller than the vector clones in the sham operated animals but this was not a significant difference (P=0.226). However, a significant (39%, P<0.001) decrease in tumor weight was observed in the antisense clones in castrated animals but not in the vector clones. In these same animals the extent of spontaneous lymph node metastasis was evaluated in terms of the number of animals with metastases (incidence) and the relative volume of the metastases as determined by computer assisted microscopic quantitation (Fig. 6B). The antisense clones had less metastatic activity compared to the vector control clones in the sham operated animals with a 17% decrease in incidence (P=0.003) and a 52% reduction in relative volume (P<0.0001). In castrated animals there was no difference in the vector control clones compared to sham operated animals however the antisense clones had a significantly further decrease in both incidence and volume of lymph node metastasis compared to the antisense clones in sham operated animals (18% and 28% respectively P<0.001). To further evaluate metastatic activity we injected cell clones directly into the tail vein and counted the number of lung metastatic deposits which formed at two weeks (Fig 6C). The antisense cav-1 clones had 40% fewer lung metastases compared to vector control clones (P<0.001).

DISCUSSION

Our previous studies have demonstrated that cav-1 is overexpressed in human and mouse metastatic prostate cancer and that overexpression of cav-1 is an independent predictor for recurrence following radical prostatectomy (3, 4). Subsequently, we demonstrated that cav-1 can protect against androgen withdrawal induced apoptosis *in vitro* and *in vivo* and that cav-1 can block c-*myc*-induced apoptosis in human prostate cancer cells (5, 9). These studies established a foundation upon which to more clearly define a role for cav-1 in prostate cancer progression. In this report we show that T upregulates cav-1 expression in prostate cancer cells in part through transcriptional regulation. We further demonstrate that increased cell viability and clonal growth *in vitro* resulting from T treatment is mediated by cav-1 protein and that modest levels but not high levels of cav-1 alone can independently

lead to increased cell viability and clonal growth. Finally, we establish that cav-1 contributes to metastasis *in vivo*.

In the first series of experiments we demonstrated that T induces cav-1 expression in part at the level of transcriptional regulation. We previously demonstrated that cav-1 is expressed at very low to nondetectable levels in normal prostate epithelium but is expressed focally in prostate cancer and further increased expression is associated with prostate cancer metastases (3-5). Together, these results suggest that T is responsible, in part, for inducing cav-1 in prostate cancer cells during progression, yet it is not clear how the cav-1 gene, which is relatively inactive in normal prostate epithelial cells, becomes responsive to T induction. Conceivably, demethylation could play a role, yet previous reports have been inconclusive regarding the role of methylation in cav-1 expression in prostate cancer and further studies are indicated (19).

To study the effects of T and cav-1 expression on prostate cancer cell survival and clonal growth activities *in vitro*, we developed a two-step assay system that mimics specific steps of metastasis *in vivo*. In the first step of this analysis, prostate cancer cells are maintained for three days at low density under serum-free conditions, mimicking the reduced growth factor and low density conditions encountered during vascular transit. Following this three-day period, cell viability was analyzed using two independent methods of analysis (MTT and ATPLite assays). Cells were subsequently seeded into a clonogenic assay, which involved a two to three-week growth period *in vitro* followed by analysis of colony number. This second step approximates growth at a distal metastatic site and is dependent on continued cell survival. The initial experiments using this assay system demonstrated that T can stimulate cell survival and clonal growth in both mouse and human prostate cancer cells.

Additional experiments in mouse prostate cancer cell lines using adenoviral vector-mediated antisense cav-1 demonstrated that cav-1 induction was responsible, in part, for T stimulated cell survival/clonal growth *in vitro*. These results are consistent, in general, with the results of our previous studies that demonstrated elevated cav-1 levels are associated with androgen insensitivity (5). In the absence of T, it is conceivable that other growth factors stimulate cav-1 expression in prostate cancer.

Others have shown that polypeptide growth factors can regulate cav-1 expression in NIH 3T3 cells (29). However, to establish a clear correlation between cav-1 expression and androgen insensitive human prostate cancer, it will be necessary to demonstrate that cav-1 expression is increased in androgen insensitive disease and to generate experimental support for androgen independent regulation of cav-1 expression in androgen insensitive prostate cancer cells. Further studies in this area are indicated.

The substitution of increased cav-1 expression via infection with AdScav-1 demonstrated that modest levels of cav-1 could also maintain viability in the assay systems described above. The results of the clonogenic assay supported and extended the results of the survival analyses indicating a several-fold increase in the number of colonies in AdScav-1-infected cells compared to control Ad-RSV-infected cells in both mouse (ABAC3) and human (LNCaP) prostate cancer cells.

The data presented in this report together with our previous studies (9) indicate that relatively modest, but not high levels of cav-1 expression can lead to increased cell viability consistent with malignant progression. Overall these results further reconcile previous reports that have shown high levels of cav-1 can suppress growth in various cell types (8, 14-17). A recent study indicates that although cav-1 is initially downregulated in colon cancer cells, re-expression of cav-1 is selected for during the development of drug resistance and metastasis (8). At the molecular level, this dichotomy between the role of cav-1 in tumorigenesis and metastasis may be explained in part by specific interactions between phosphorylated cav-1 and downstream signaling molecules (30). Further studies are required to define the molecular mechanism(s) through which cav-1 specifically promotes survival/clonal growth in prostate cancer cells.

Finally we generated *in vivo* data that support our *in vitro* studies and demonstrate that experimental reduction of cav-1 expression results in suppression of metastatic activities *in vivo*. Using stable antisense mouse prostate cancer cell clones, our results indicate that both spontaneous and experimental metastatic activities can be significant reduced by the suppression of cav-1 expression. The results of our castration studies further suggest that the presence of circulating testosterone together with cav-1 can produce synergistic effects that increase metastatic activities. Interestingly, although a reduction

of cav-1 levels activities suppressed metastatic activities it did not suppress primary tumor growth demonstrating that the effects of cav-1 *in vivo* are metastasis specific in this prostate cancer model.

Our results have demonstrated that T can induce cav-1 expression in part through transcriptional regulation and that cav-1 overexpression is, in part, responsible for T-stimulated survival of mouse prostate cancer cells *in vitro*. Further studies documented that modest, but not high levels of cav-1 can support survival of prostate cancer cells under pro-apoptotic conditions, i.e., growth factor depletion and low cell density, and promote clonal growth *in vitro*. Finally, analysis of spontaneous and experimental metastasis using stably transfected antisense cav-1 prostate cancer cells confirmed that elevated cav-1 levels contribute to metastasis of prostate cancer cells *in vivo*. Further studies will be needed to define the molecular mechanism(s) through which cav-1 contributes to prostate cancer metastasis.

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FIGURE LEGENDS

Fig. 1. T significantly enhances cell survival of androgen-sensitive prostate cancer cells under conditions of growth/survival factor depletion. A) ABAC3 (open bar) and LNCaP (filled bar) cells were seeded at low density (200 cells/well in 96-well plate) and incubated for 3 days in SFM or SFMT with various concentration of T. Cells were stained with MTT then live and dead cells were microscopically counted. The viability was expressed as fraction of viable cells (viable cells/total cells) and normalized to control (control = 1; T = 0 nM was used as control). *B)* ABAC3 (open bar) and LNCaP (filled bar) cells were seeded in SFM or in SFMT with various concentration of T in 12-well plates (1×10^4 cell/well for ABAC3 and 1×10^5 cells/well for LNCaP) for 3-days. ATP levels were used as indicator of cell viability. Light units generated by ATP in each sample were normalized by that of control (T= 0) and expressed as relative ATP level. *C)* ABAC3 and LNCaP cells were pretreated with SFM (open bar) or SFMT (filled bar) (T= 20 nM for ABAC3 and 5 nM for LNCaP) in 96-well plates for 3 days, trypsinized and reseeded in 10-cm plates with complete media for 10-15 days till colonies were stained and counted. Error bar, standard deviation. *, significance of $P < 0.05$. **, significance of $P < 0.0001$ compared to T=0.

Fig. 2. Dose-dependent induction of caveolin-1 protein by T. Cells were seeded at low density (2.0×10^5 cells/15-cm plate) and grown in SFM or SFMT with indicated concentrations of T. Cell lysates were prepared after 2 days and cav-1 and β-actin detected on western blots.

Fig. 3. Transcriptional up-regulation of caveolin-1 by T. The 721 bp mouse cav-1 promoter-luciferase reporter (mCav-1) or its control pGL3-basic luciferase reporter (Basic) was cotransfected with pCMV-β-gal into ABAC3 or LNCaP cells. Following 48h treatment in SFM (open bar) or SFMT (filled bar, T= 20 nM for ABAC3 and 10 nM for LNCaP) or SFMT with 1 μM casodex (hatched bar) cell lysates were

prepared and reporter activities determined. Error bar, standard deviation. **, significance of $P < 0.0001$ compared to SFM.

Fig. 4. Antisense cav-1 significantly inhibits survival activities mediated by T. ABAC3 cells were double-infected with a adenoviral vectors at MOI of 25 or 50 then split to SFM or SFMT (T= 20 nM). Un = uninfected, R = control vector AdRSV, AS = AdAScav-1. *A)* The expression of cav-1 and AR protein was determined by western blotting after 2 days. *B)* Viability of cells determined by MTT assay after 3 days in SFM (open bars) or SFMT (filled bars). *C)* After 3 days in SFM (open bars) or in SFMT (filled bars), cells were trypsinized and reseeded in 10-cm plates for the clonogenic assay. Error bar, standard deviation. *, significance of $P < 0.05$. **, significance of $P < 0.0001$ (compared to SFM).

Fig. 5. Cav-1 expression alone accounts for a significant component of the survival activities in LNCaP cells. *A)* Western blot showing dose-dependent expression of cav-1 mediated by adenovirus expressing human cav-1. (RSV= control vector AdRSV, Scav = sense human cav-1 vector AdScav-1, AS = antisense human cav-1 vector, the number following R or S = MOI). A cav-1 dose-dependent viability protection following growth/survival factor depletion was demonstrated by the MTT assay *(B)*, the ATPLite assay *(C)* and the clonogenic assay *(D)*. Symbols in D, AdScav-1 (filled bars) and vector control AdRSV (open bars). Immunostaining of cav-1 in uninfected LNCaP cells *(E)* or in AdScav-1 infected LNCaP cells (MOI = 25) (**F**). Error bar, standard deviation. *, significance of $P < 0.05$. **, significance of $P < 0.0001$.

Fig. 6. Antisense cav-1 suppresses prostate cancer metastasis *in vivo*. *A*. Analysis of orthotopic tumor wet weight for vector control clones with sham surgery (V/Sh), antisense cav-1 clones with sham surgery (AS/Sh), vector control clones with surgical castration (V/Cas), and antisense cav-1 clones with surgical castration (AS/Cas). Each clone was evaluated in eight to nine animals and tumor weight compared to V/Sh. *B)*. The number of animals in each group with microscopic evidence of lymph node metastasis, incidence for the four groups of animals in A is depicted by open bars. The relative extent of metastatic

infiltration of the lymph nodes as measured by computer assisted analysis was compared to the V/Sh group (filled bar). *C)* Number of lung metastatic deposits, counted microscopically, resulting from tail vein inoculation of vector or antisense clones in the experimental metastasis assay.

Error bar, standard error. *, significance of $P < 0.05$. **, significance of $P < 0.0001$.

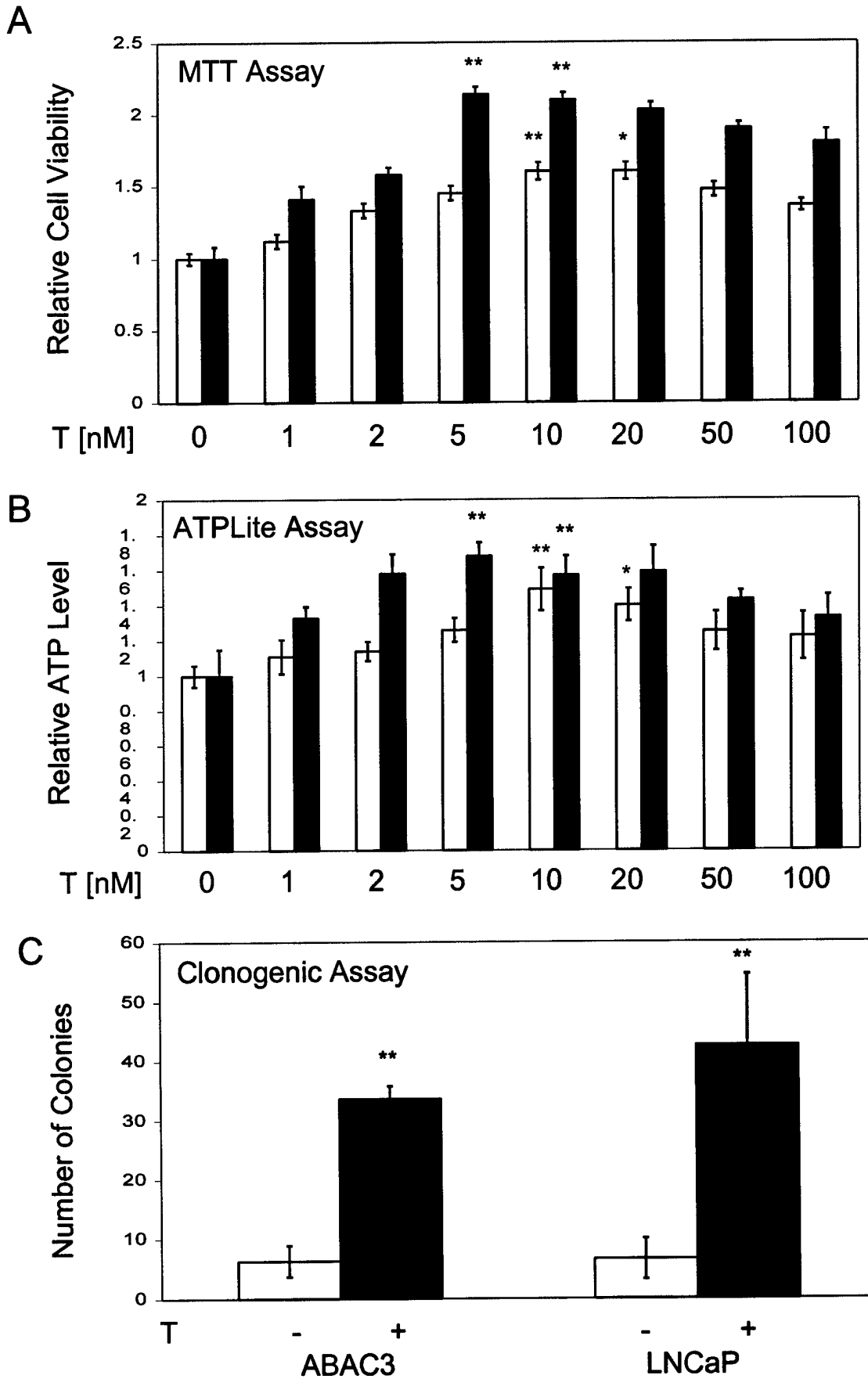

A
MTT Assay
Relative Cell Viability
2.5
2
1.5
1
0.5
0
T [nM]
0
1
2
5
10
20
50
100
B
ATPLite Assay
Relative ATP Level
C
Clonogenic Assay
Number of Colonies
60
50
40
30
20
10
0
T
-
+
ABAC3
LNCaP

Figure 2

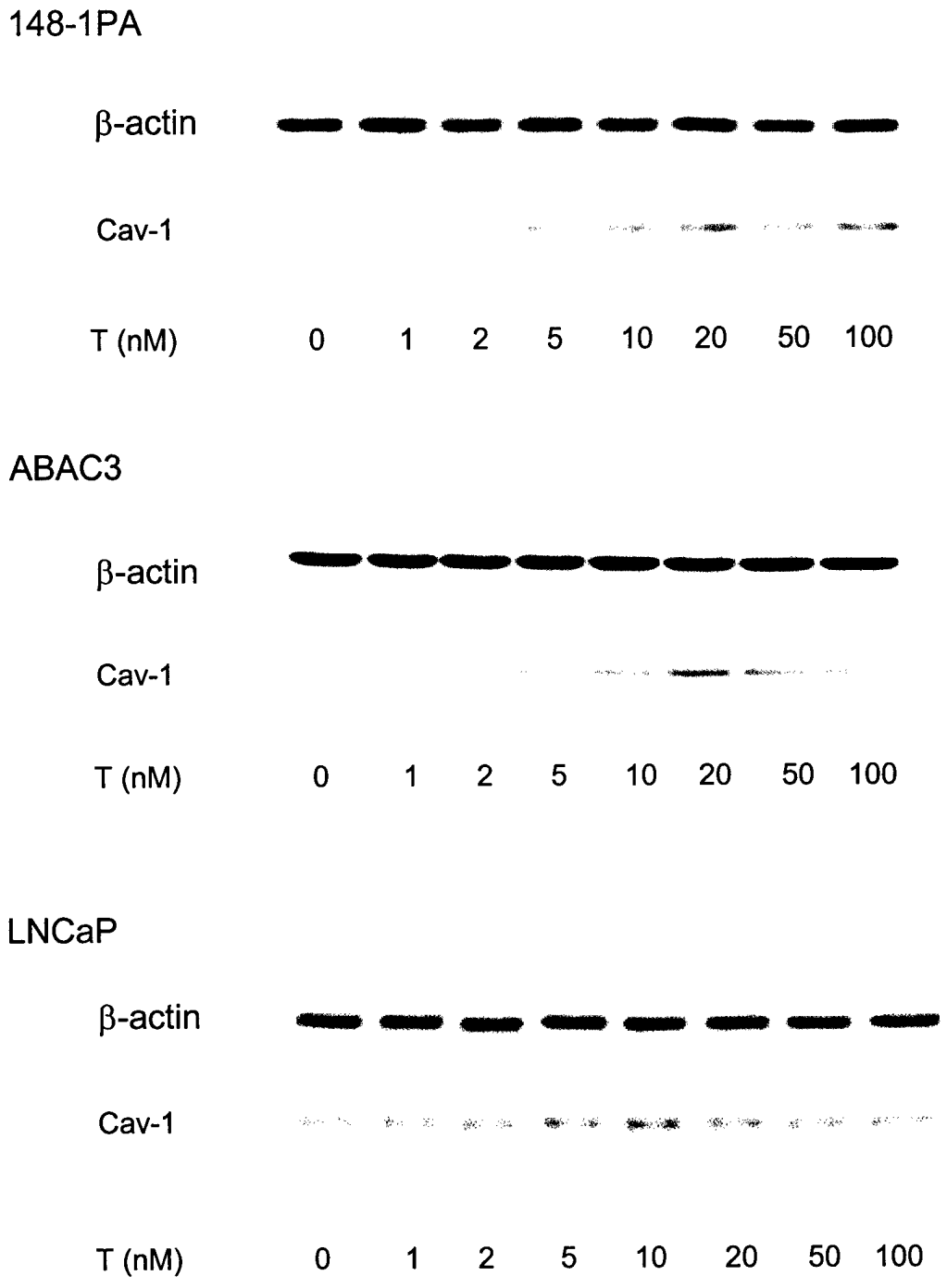

148-1PA
β-actin
Cav-1
T (nM)
0 1 2 5 10 20 50 100
ABAC3
β-actin
Cav-1
T (nM)
0 1 2 5 10 20 50 100
LNCaP
β-actin
Cav-1
T (nM)
0 1 2 5 10 20 50 100

Figure 3

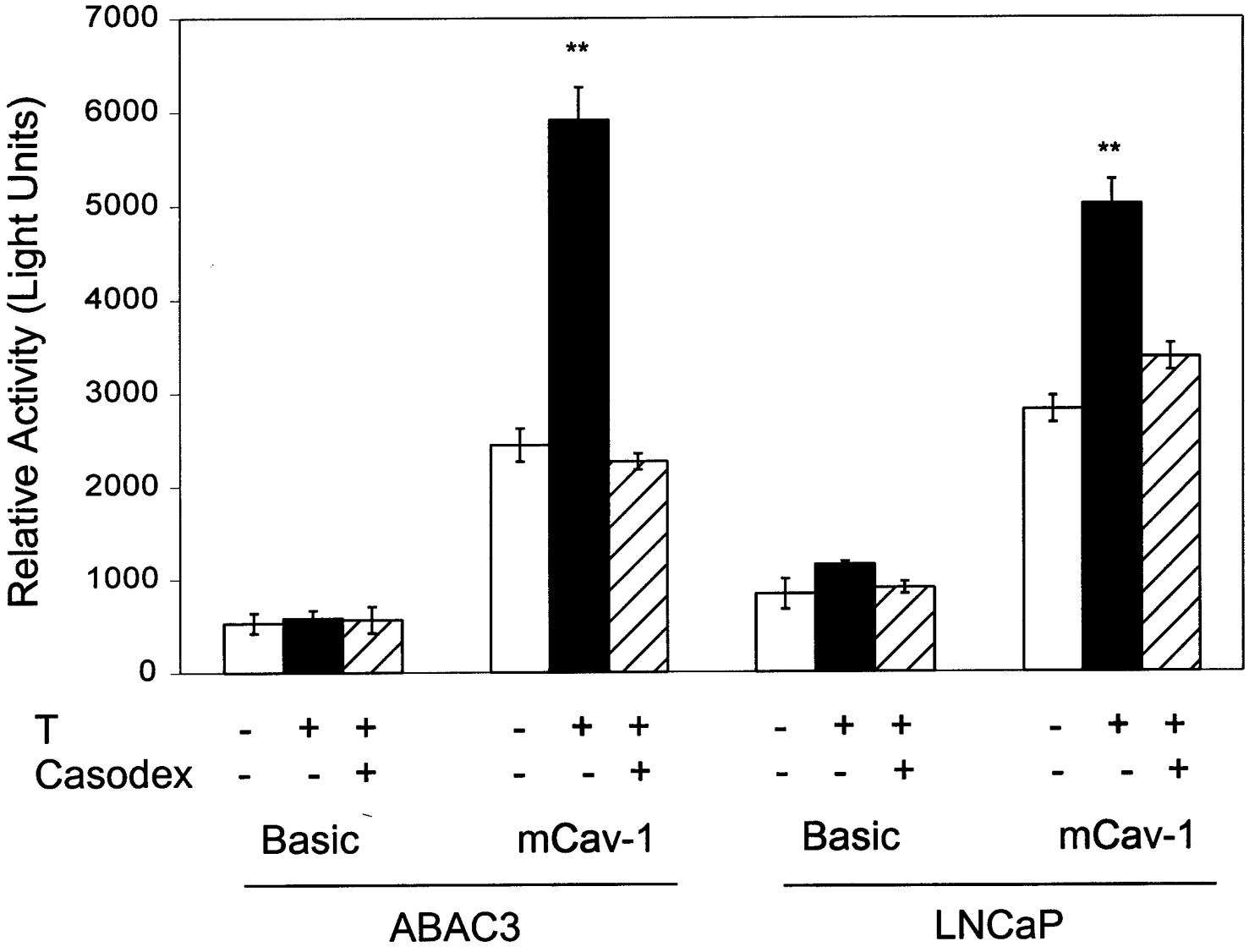
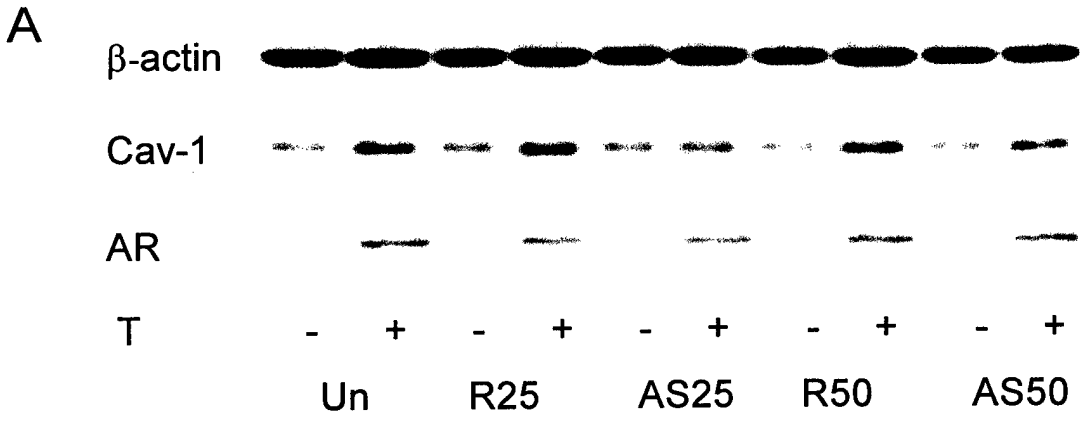
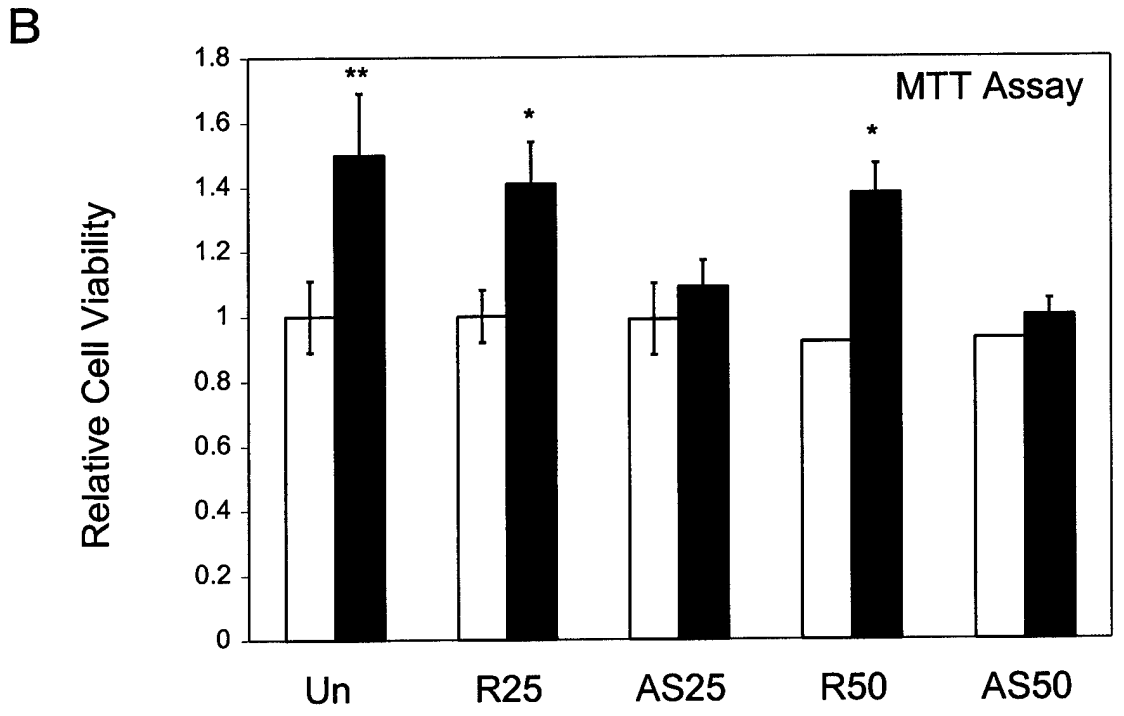

Relative Activity (Light Units)
7000
6000
5000
4000
3000
2000
1000
0
**
**
T
Casodex
- + +
- - +
- + +
- - +
- + +
- - +
- + +
- - +
Basic
mCav-1
Basic
mCav-1
ABAC3
LNCaP

Figure 4


A
β-actin
Cav-1
AR
T
- + - + - + - + - +
Un
R25
AS25
R50
AS50


B
MTT Assay
Relative Cell Viability
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
*
Un
R25
AS25
R50
AS50

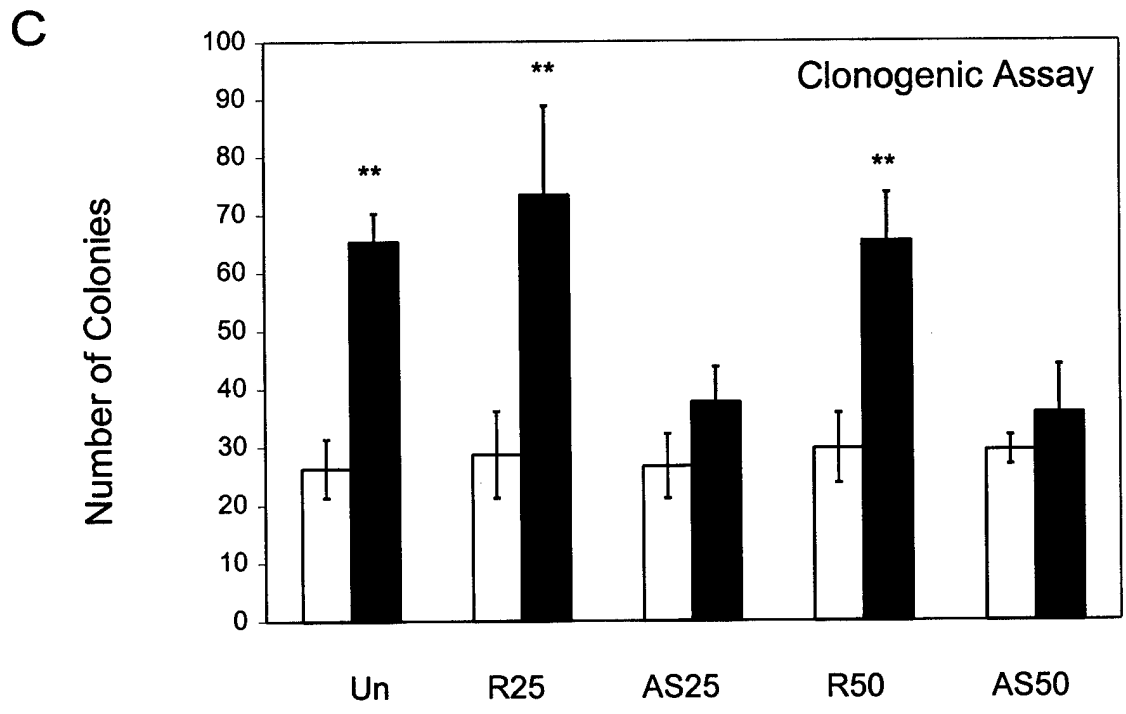
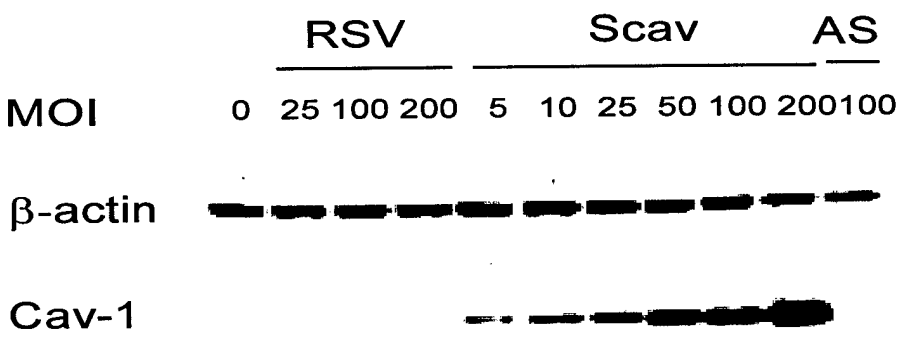
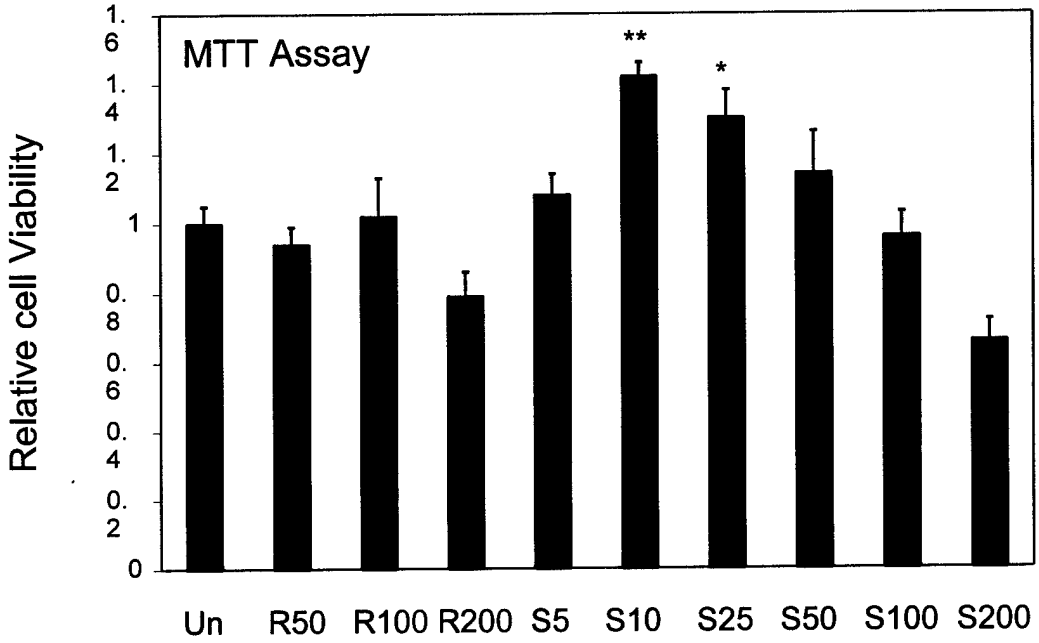

C
Clonogenic Assay
Number of Colonies
100
90
80
70
60
50
40
30
20
10
0
**
**
**
Un
R25
AS25
R50
AS50

Figure 5A-C

A



B



C

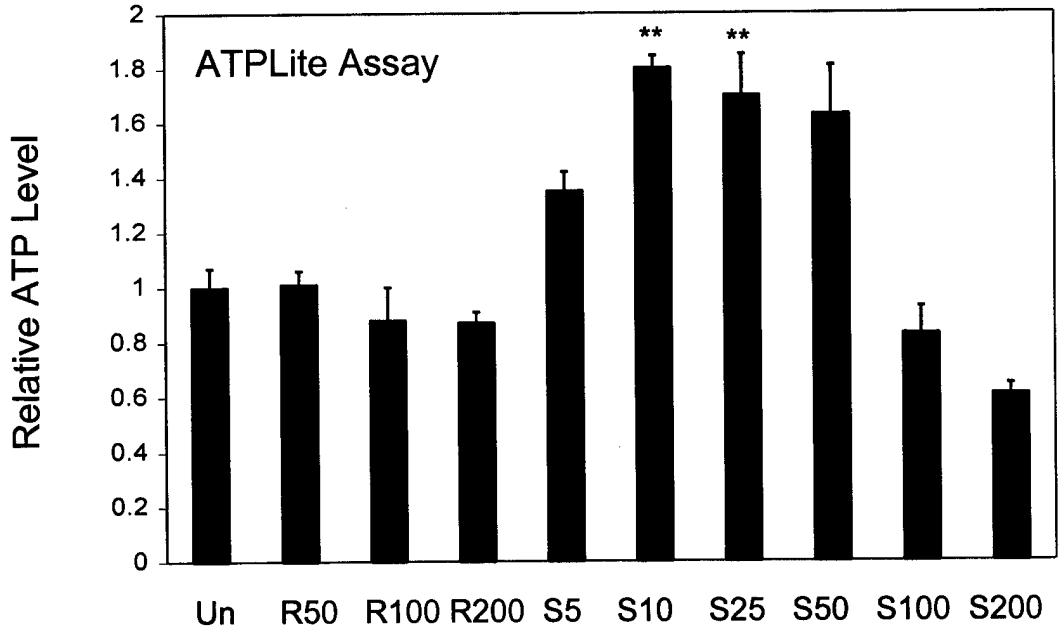
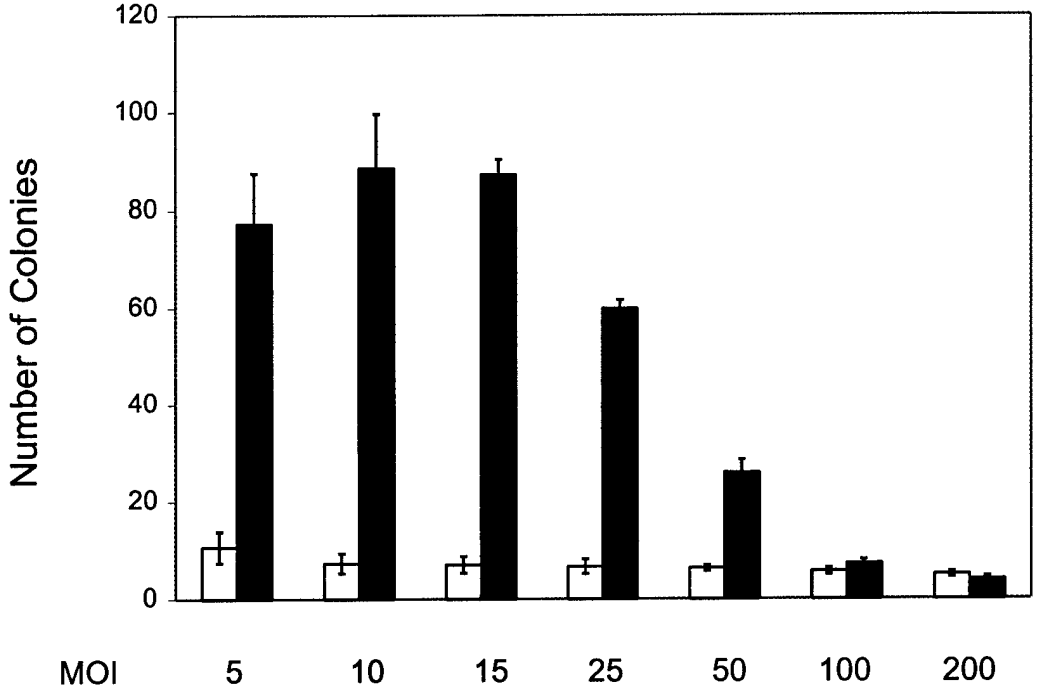


Figure 5D-F

D



E

F

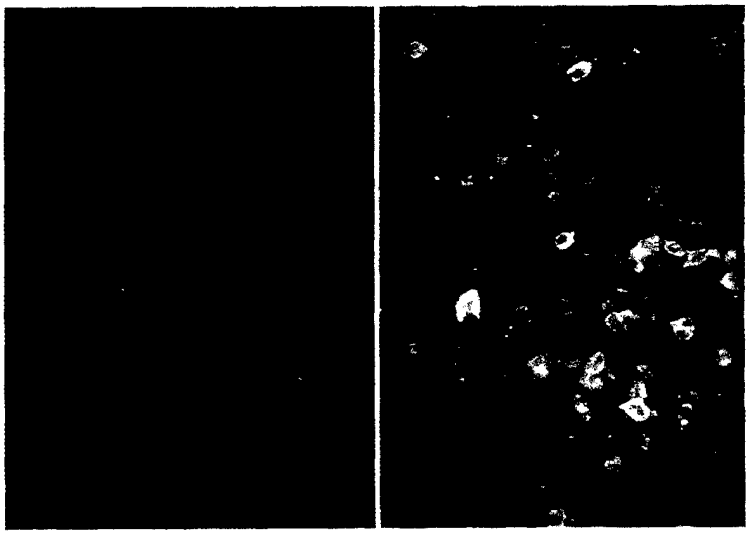
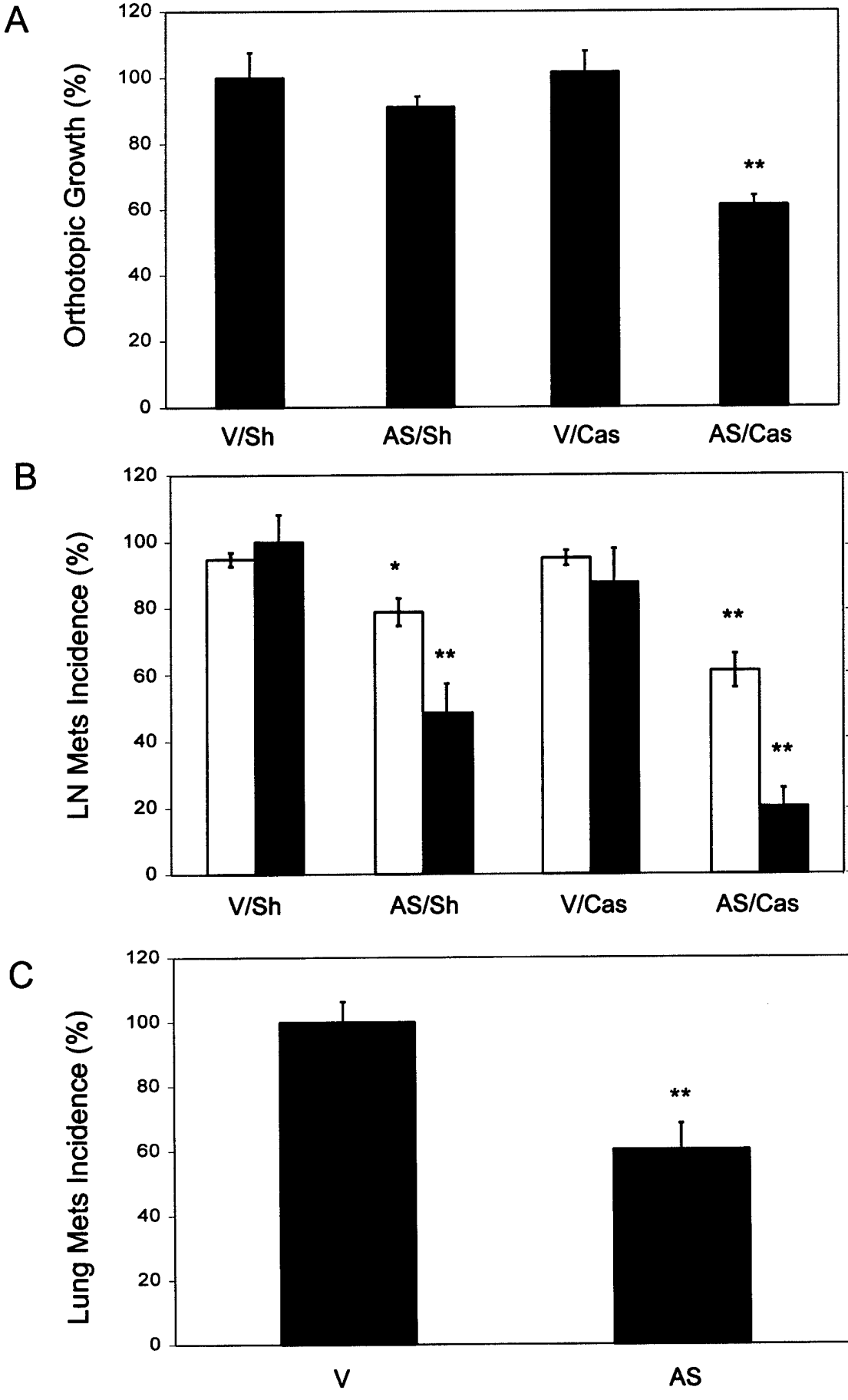


Figure 6


A
Orthotopic Growth (%)
120
100
80
60
40
20
0
**
V/Sh
AS/Sh
V/Cas
AS/Cas
B
LN Mets Incidence (%)
LN Mets Volume (%)
120
100
80
60
40
20
0
12 0
10 0
80
60
40
20
0
*
**
**
**
V/Sh
AS/Sh
V/Cas
AS/Cas
C
Lung Mets Incidence (%)
120
100
80
60
40
20
0
**
V
AS